Root and Butt Rots of Forest Trees

10th International Conference on Root and Butt Rots

Proceedings of the IUFRO Working Party 7.02.01
Québec City, Canada, September 16-22, 2001

G. Laflamme, J.A. Bérubé, G. Bussières (éditeurs/editors)

Centre de foresterie des Laurentides - Laurentian Forestry Centre
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(Droite) Fructifications de *Heterobasidion annosum* sur une souche de pin rouge (C. Moffet, SCF)

Cover photos:
(Left) Circular patch of dead red pines killed by *Heterobasidion annosum* (R. Blais, CFS)
(Right) *Heterobasidion annosum* fruiting bodies on a red pine stump (C. Moffet, CFS)

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FOREWORD

IUFRO Working Party 7.02.01 usually convenes every four years. The 10th International Conference on Root and Butt Rot was held in Quebec City, Canada, from September 16–21, 2001. The event attracted 67 participants from 13 countries, although 29 members were unable to attend due to the tragic events of September 11, 2001. Gaston Laflamme, the conference organizer, and Ariane Plourde, Research Director at the Laurentian Forestry Centre, Canadian Forest Service, welcomed the delegates. Working Party co-ordinator Claude Delatour chaired the business portion of the meeting, in which it was decided that Europe, possibly Poland, would be the site of the next meeting.

After the scientific communication sessions, participants travelled to the Montreal and Ottawa regions for field trips. The delegates visited forests infested by *Inonotus tomentosus*, *Heterobasidion annosum* and *Armillara* sp.

The proceedings contain the texts or abstracts of papers submitted to the organizing committee and of the 55 scientific posters presented at the meeting. They also include texts by those who were registered for the conference but who could not attend. Authors are responsible for content. Texts are presented under the following six headings: "Phylogeny and Taxonomy", "Ecology and Biodiversity", "Control", "Genetics and Population Dynamics", "Pathogenicity, Resistance and Etiology" and "Incidence and Epidemiology".

Gaston Laflamme, Conference organizer
Co-ordinator, IUFRO WP 7.02.01

Jean Bérubé and Guy Bussières, Conference co-organizers

Conference Website: www.cfl.scf.rncan.gc.ca/iufro-rbr2001

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We would also like to express our appreciation to the field trip organizers, particularly Robert Blais and Julie Dubé (CFS–LFC), Louise Innes and Solange Simard (MRNQ), Mike Dumas (CFS–GLFC) and John McLaughlin (OMNR).

In addition, we are grateful for the assistance provided by the staff of Communications Services, CFS–LFC. We thank Charles-Paul Coulombe and Benoit Arsenault, who created and updated the conference's Web site with the help of the LFC's Informatics Services team, and we thank the editing team made up of Isabelle Lamarre, Diane Paquet and Pamela Cheers, who took on the arduous chore of producing this document and without whom the publication of these proceedings would have been very difficult indeed.

G. Laflamme, J.A. Bérubé and G. Bussières

AVANT-PROPOS

Le groupe de travail 7.02.01 de l'IUFRO se réunit habituellement à tous les quatre ans. C'est dans la ville de Québec, Canada que s'est tenu la 10^e Conférence sur la pourridiés des arbres forestiers, du 16 au 21 septembre 2001. L'événement a regroupé 67 participants provenant de 13 pays, bien que la tragédie du 11 septembre 2001 nous ait privés de la participation de 29 membres. Les délégués ont été accueillis par Gaston Laflamme, organisateur du congrès, et par madame Ariane Plourde, directrice de la recherche au Centre de foresterie des Laurentides du Service canadien des forêts. Une session d'affaires du groupe de travail s'est tenue sous la présidence de M. Claude Delatour, coordonnateur du groupe de travail; parmi les différents sujets de discussion, il a été décidé de privilégier l'Europe comme site de la prochaine réunion, qui serait possiblement tenue en Pologne.

Après les sessions des communications scientifiques, les congressistes se sont rendus dans les régions de Montréal et d'Ottawa pour participer à des excursions en forêt. Ils ont eu l'occasion de visiter principalement des stations affectées par *Inonotus tomentosus*, *Heterobasidion annosum* et *Armillaria* sp.

Le compte rendu regroupe les textes ou les résumés des communications soumis au comité organisateur et ceux des 55 affiches scientifiques présentées pour la réunion de ce groupe de travail. Les textes de ceux et celles qui étaient inscrits au congrès mais qui n'ont pu se rendre sur les lieux de la conférence sont intégrés au document. Il est à noter que les auteurs sont responsables du contenu de leurs textes. Les textes sont regroupés sous les six thèmes suivants : « Phylogénie et taxonomie », « Écologie et biodiversité », « Contrôle », « Génétique et dynamique des populations », « Pathogénicité, résistance et étiologie », ainsi que « Incidence et épidémiologie ».

Gaston Laflamme, organisateur de la conférence
Coordonateur du IUFRO WP 7.02.01

Jean Bérubé et Guy Bussières, co-organisateurs de la conférence

Site Web de la conférence : www.cfl.scf.rncan.gc.ca/iufro-rbr2001

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Il nous faut souligner de façon particulière tous ceux et celles qui ont pris part à l'organisation des visites de terrain, en particulier Robert Blais et Julie Dubé, SCF-CFL, Louise Innes et Solange Simard, MRN-Québec, Mike Dumas, SCF-CFGL, et John McLaughlin, MRN-Ontario.

Enfin, nous avons grandement apprécié l'aide du personnel des Services des communications du SCF-CFL. Nos remerciements vont à Charles-Paul Coulombe et Benoit Arsenault qui, avec l'équipe des Services informatiques du CFL, ont créé et mis à jour le site Web du congrès, ainsi qu'à l'équipe d'édition constituée d'Isabelle Lamarre, Diane Paquet et Pamela Cheers, qui ont su mener à terme ce long travail d'édition et sans qui ce compte rendu aurait été difficilement réalisable.

G. Laflamme, J.A. Bérubé et G. Bussières

Phylogeny and Taxonomy

PRELIMINARY CHARACTERIZATION OF *ARMILLARIA* ISOLATES FROM TEA (*CAMELLIA SINENSIS*) IN KENYA

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SUMMARY

The taxonomy of *Armillaria* in several African countries remains unresolved, but *A. heimii* and *A. mellea* are the two main species described in Kenya. A survey covering the main tea growing districts in Kenya was carried out in 1997 for the presence of *Armillaria* spp. and 47 isolates were collected from infected tea plants. Cultural morphology, somatic incompatibility reactions, PCR-RFLPs of ITS and IGS and DNA sequencing of the IGS were performed for the characterization of the collected isolates. For comparison purposes, Kenyan isolates of *A. mellea* (K5 and K8) and Kenyan isolates K10 and K12 (belonging to a yet unnamed biological species) were selected as reference isolates. The isolates were separated into two groups by morphology and somatic incompatibility. The restriction pattern of the ITS using *Alu* I, *Hinf* I, and *Nde* II and the restriction pattern of the IGS using *Alu* I confirmed the morphological grouping. Group I may represent *A. heimii* and group II could be a new species. The latter was supported by the sequencing of the IGS. Comparison of the sequences with those published in the Genbank database showed that they were different from *A. mellea* and identical to K10 and K12. No *A. mellea* was found during the survey.

Keywords: *Armillaria*, IGS, ITS, phylogeny, Kenya

INTRODUCTION

Tea (*Camellia sinensis*) is a crop of great significance in Kenya and *Armillaria* is a primary pathogen causing root rot. It is one of the most important diseases of tea and the losses in small tea farms can be as high as 50% (Onsando et al. 1997). The losses in larger farms are lower due to disease control programs. There are two *Armillaria* species recorded in this country, *A. mellea* (Vahl) Kummer and *A. heimii* Pegler. Ota et al. (2000) confirmed by isozyme and RAPD analysis that isolates of *A. mellea* in Africa were identical to isolates of *A. mellea* from Japan, they derived from the same origin and migration may have occurred from Asian countries to Africa.

The identification of African *Armillaria* species is often limited by the absence or scarcity of basidiomata in tropical regions (Gibson 1960). The lack of basidiomata and haploid testers has led to the identification of the *Armillaria* species based on vegetative mycelia, and in the field the disease is mainly confirmed by the white mycelium found underneath the bark of infected roots.

Different methods have been used for the identification of *Armillaria* in Africa based on somatic incompatibility (Abomo-Ndongo and Guillaumin 1997), isozyme electrophoresis (Agustian et al. 1994; Mwangi et al. 1989; Mwenje and Ride 1996, 1997), molecular markers such as DNA restriction fragment polymorphisms (Anderson et al. 1987; Chillalli et al. 1997; Smith and Anderson 1989) and DNA sequence analysis (Anderson and Stasovski 1992). Mohammed (1994) used RAPD markers to distinguish African isolates with diverse origins. RFLP and nucleotide sequence data of the intergenic spacer region of the ribosomal DNA operon were recently used to distinguish between Southern Africa isolates of *Armillaria* (Coetzee et al. 1997, 2000). The authors showed that both nuclear and organelle DNA-based molecular markers provide an alternative to mating tests and basidiomata morphology that can aid systematics of *Armillaria* in Africa.

Identification of *Armillaria* species in some African countries remains unresolved and this was highlighted by Coetzee et al. (2000) who studied *Armillaria* in pine plantations in South Africa where *A. mellea* and *A. heimii* were also described. They concluded in their research that isolates identified as *A. heimii* were in fact *Armillaria* sp. or *A. fuscipes* (Petch 1909).

The objective of this study was to characterize the *Armillaria* species affecting tea in Kenya using different methods based on morphology, somatic incompatibility, PCR-RFLPs of the ITS and IGS region and sequence of the IGS region.

MATERIALS AND METHODS

Isolates

In 1997, 14 districts were surveyed in the main tea growing areas in Kenya. Forty-seven isolates of *Armillaria* were collected mainly from infected roots of tea (*Camellia sinensis*), but also *Dombeya* sp., *Dioscorea* sp., *Coffea arabica*, *Musa acuminata* and *Eucalyptus* sp. African isolates of *A. mellea* (K5, K8 and ST1) and isolates K10 and K12 from Kenya were donated by Dr J.-J. Guillaumin (INRA Clermont-Ferrand, France) for comparison purposes.

Basidiomata and cultural morphology

Morphological features of basidiomata were recorded when these were present. Production of basidiomata was attempted *in vitro* in 1 litre flasks. The medium consisted of 50 g of milled beech wood sawdust, 20 g of whole blended orange, 60 g of whole grain rice, 10 g of peptone and 5 g of agar (Tirro 1991). The mixture was topped up with 400 ml of water and autoclaved for 1 hour at 121°C. The flasks were inoculated and incubated at 25°C for 4 weeks in the dark. After this period the temperature was adjusted at 20°C with a 12 hour photoperiod.

The isolates were cultured on 2% MEA and 3% MEA with added peptone (0.06%). The plates were incubated in the dark at 22°C over four weeks and cultural morphology was described.

Somatic incompatibility

Isolates were paired as 3 mm plugs on 2% MEA overlaid with cellophane using the method described by Hopkin et al. (1989). The plates were incubated in the dark for three weeks at 20°C. After this time, a 2 x 2 cm square of cellophane was cut out containing the paired isolates and immersed in freshly prepared L-Dopa solution (0.05%). These were incubated at 37°C for 1 hour before being examined for the presence of the black line between the thalli (Mallet et al. 1986).

DNA extraction

The isolates were grown in liquid culture (1% malt extract, 0.5% yeast extract and 1% glucose) and incubated in the dark for three weeks at 20°C. The flasks were not shaken during this time. The mycelium was harvested, rinsed with distilled water, frozen in liquid nitrogen and stored at -80 °C. The DNA was extracted from the frozen mycelium using a Dneasy^{TM} Plant Mini Kit (Quiagen).

PCR-RFLPs

The internal transcribed spacer (ITS) was amplified by PCR with the universal primers ITS1 and ITS4 (White et al. 1990). The intergenic spacer (IGS) region between the 26S and 5S was amplified with two different sets of primers. The first set included LR12R, 5' CTG AAC GCC TCT AAG TCA GAA 3' (Veldman et al. 1981) and O-1, 5' AGT CCT ATG GCC GTG GAT 3' (Duchesne and Anderson 1990) recommended by Anderson and Stasovski (1992). The second set of primers included: P-1, 5' TTG CAG ACG ACT TGA ATG G 3' and 5S-2B, 5' CAC CGC ATC CCG TCT GAT CTG CG 3' recommended by Coetzee et al. (1997). Ready-To-Go PCR beads (Amersham Pharmacia Biotech) were used for the PCR amplification. Individual reactions were brought to a final volume of 25 µl. Each reaction contained 1.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, stabilizers including BSA, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.1 µM of each primer and purified water (Sigma Chemical Co.). The PCR amplification program to amplify the ITS was as described by Chillali et al. (1997). The PCR program to amplify the IGS region consisted of 1 cycle of 95°C for 95 sec, followed by 35 cycles of 60°C for 60 sec, 72°C for 120 sec and 95°C for 60 sec and a final extension at 72°C for 10 min. The amplifications were performed on a Progene (Techne, UK) thermocycler. The ITS and IGS amplified products were purified with a QIAquick ™ Purification Kit (Quiagen). The ITS was digested separately with 5 units of the restriction enzyme *Hinf* I, *Alu* I and *Nde* II and the IGS was digested with 5 units of *Alu* I. The restriction patterns were visualized in 3% agarose gels stained with ethidium bromide.

Sequencing of the IGS was carried out by MWG Biotech. Lasergene (DNASTAR 2000) software for Macintosh was used for editing and aligning the sequence files. Additional sequences from the GenBank databases available through the National Center for Biotechnology Information (NCBI, Bethesda, MD) were obtained. The alignments were made with Megalin and the indels coded using MacClade (Maddison and Maddison 1992). Phylogenetic analyses were performed using PAUP version 4.0b (Swofford 1998)

RESULTS

Basidiomata and cultural morphology

Basidiomata were only present in one location (Kericho) at >2180 m altitude during the rainy season. They appeared in clusters fused at the base. The pilei were 8.5-16.5 mm in diameter, convex, applanate to umbonate, with a non-striate margin, light ochraceous but dark-brown at the disk centre. The stipe was creamy-white in colour, 45-50 x 3-6 mm with a whitish, fugacious annulus attached to the upper quarter of the stipe. Basidia were 30-35 x 6-7 µm, elongate clavate with four sterigmata. Lamellae were white in colour. The basidiospores were sub-globose to ovoid, 4.5-7.5 x 4-6.5 µm. Clamp connections were absent. Only very immature basidiomata were obtained in the *in vitro* attempts.

The isolates were separated into two groups based on morphology in culture. Group I consisted of isolates whose colonies were mainly rhizomorphic and only mycelium was observed at the centres. Group II consisted of isolates which had raised mycelial colonies with submerged rhizomorphs.

Somatic incompatibility

A reaction was considered incompatible when a pigmented line was observed at the interfaces and compatible when the colonies merged. Two different groups were found which corresponded with the two morphological groups. Isolates from group I were compatible among themselves and incompatible with group II. Isolates from group II were incompatible both with isolates from group I and with each other. Isolates from group II when paired against each other showed a clear and distinct pigmented line consisting of melanized hyphae.

PCR-RFLPs

The ITS region of isolates from group I by cultural morphology was amplified and a PCR product of about 700 bp was obtained. A PCR product of about 900 bp was amplified for isolates in group II. The IGS region of isolates in group I was amplified with the primers P-1 and 5S-2B and gave a PCR product of over 1000 bp. The IGS region of isolates in group II was amplified with the primers LR12R and O-1 and gave a PCR product of about 800 bp. A similar band was obtained for the isolates of *A. mellea* K5, K8 and ST1 and isolates K10 and K12. The digestion of the ITS of group I with the restriction enzyme *Hinf* I gave for group I a pattern of about 220, 190, 170 and 72 bp and for group II a pattern of about 360, 230, 150, 100 bp (Figure 1a).

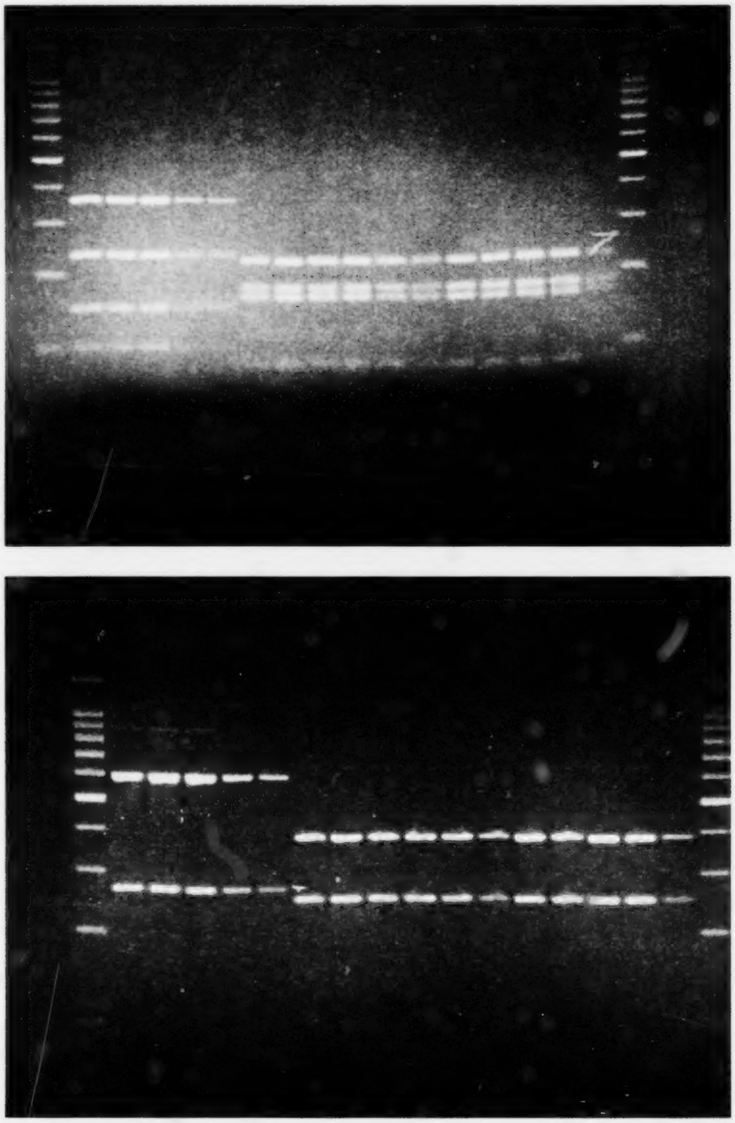

Figure 1 (a, b). Digestion of ITS region with *Hinf* I (fig 1a) and with *Nde* II (fig 1b) on 3% agarose gel stained with ethidium bromide. Lanes 2-6: isolates from group II. Lanes 7-17: isolates from group I. A 100bp ladder was used as a size marker in lane 1 and lane 18.

The digestion of the ITS with restriction enzyme *Alu* I gave for group I a pattern of about 480, 160, 85 bp and for group II a pattern of about 510, 225, 95 bp. The digestion of the ITS with restriction enzyme *Nde* II for group I gave a pattern of about 390, 250 bp and for group II a pattern of about 590, 270 bp (Figure 1b).

The digestion of the IGS with the restriction enzyme *Alu* I gave three different patterns, for group I a pattern of about 380, 245, 135 bp was obtained, for group II a pattern of 310, 220, 135 bp was obtained and for *A. mellea* a pattern of about 310, 170, bp was obtained. Isolates K10 and K12 had similar restriction patterns that group II (Figure 2).

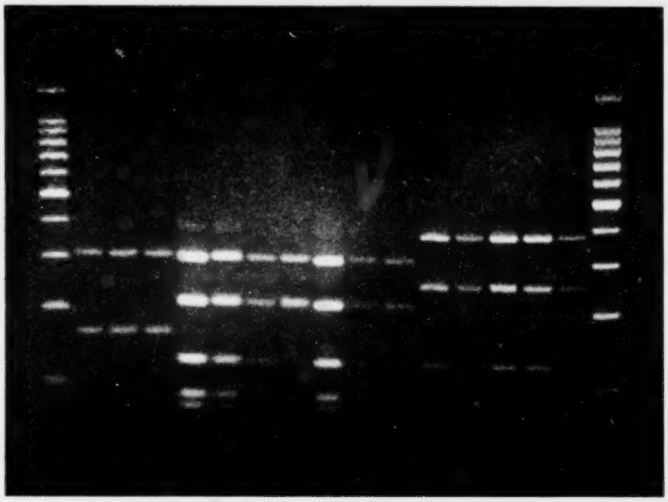

Figure 2. Digestion of IGS region with *Alu* I. on 3% agarose gel stained with ethidium bromide. Lanes 2-4: isolates K5, K8 and ST1. Lanes 5-9: isolates from group II. Lanes 10-11: isolates K10 and K12. Lanes 12-16: isolates from group I. A 100 bp ladder was used as a size marker in lane 1 and lane 17.

The phylogenetic studies of the IGS region were performed only for group II. The results showed that all the isolates from group II and isolates K10 and K12 formed one clade (100% Jackknife support) that was separated from the group of *A. mellea* also supported by a 100% Jackknife value. The isolate K5 (*A. mellea* from Kenya) was grouped with isolates of *A. mellea* from Japan and South Korea supporting Ota et al. (2000) theory. The clade support for these clusters had 100% Jackknife value.

DISCUSSION

Cultural morphology and somatic incompatibility separated the 47 isolates into two groups. Group I with rhizomorphic colonies and group II with mycelial colonies and submerged rhizomorphs. Molecular data based on the ITS and IGS regions separated the isolates into the same two groups.

Basidiomata were only found once and they corresponded to group I. The description of the basidiomata conforms to that of *A. heimii* (Heim 1963; Pegler 1977) except for the stipe size which was slightly larger compared to the original description (2.5-4.5 x 2-3 mm). The cultural morphology of the isolates in this group also resembles *A. heimii* (Mwangi, pers. comm.). The amplification of the ITS region by PCR gave a product of about 700 bp that was similar to the size described by Chillali et al. (1997) for *A. heimii* and the restriction pattern with the enzyme *Nde* II was similar, but the restriction patterns with the enzymes *Alu* I and *Hinf* I were completely different from the ones obtained for *A. heimii*. The IGS region of group I was amplified and a band of over 1000 bp was obtained. This PCR product was different in size from *A. heimii* from other African countries. The digestion with the restriction enzyme *Alu* I gave a different pattern to the *A. heimii* but was similar to the one obtained by Coetzee et al. (2000) and identified as *Armillaria* sp. or *A. fuscipes*. Even though the morphological data point to *A. heimii*, molecular data suggested that there are sub-groups within *A. heimii* or they are a complex of several species.

No basidiomata were found in nature for group II. This group was different from group I and different from *A. mellea*. This was supported by the PCR-RFLP results obtained for the ITS and IGS regions. Isolates from this group were identical to isolates K10 and K12 previously described as potential new *Armillaria* species (Chillali et al. 1997). The phylogenetic analysis showed that the IGS region between the 26S and 5S of the isolates from group II was identical to the IGS region of isolates K10 and K12 and different from *A. mellea* and other *Armillaria* species. The IGS sequence of group II was different from any other *Armillaria* sequence published in GenBank. The data suggest that group II could be a new species but basidiomata are essential for the complete description and naming of the species. So far only very immature basidiomata have been produced in the *in vitro* attempts.

Somatic incompatibility is one of the methods that has been used for the identification of genotypes and the incompatible reaction is characterized by the presence of a black line along the demarcation zone. The isolates from group II showed this reaction when paired against each other. The black line is usually absent in pairings between two genotypes of the same species (Guillaumin et al. 1991). This phenomenon has been reported for African *Armillaria* by Abomo-Ndongo (1997). A similar phenomenon has been observed for *Ganoderma* in oil palms where most isolates, even when taken from the same plant, were somatically incompatible with one another (Miller et al. 1999). Care should therefore be taken in interpreting somatic compatibility tests aimed at delimiting species in *Armillaria*.

It can be concluded from this study that two different *Armillaria* species were found affecting tea plantations. One is suspected to be *A. heimii* and the other a possibly new *Armillaria* species. It was surprising that no isolates conforming to *A. mellea* were found and this may be an indication that its importance in Africa has been overestimated. More research is needed to resolve the taxonomy of *Armillaria* species in Kenya.

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PHYLOGENETIC RELATIONSHIP AMONG *LAETIPORUS* SPP. IN JAPAN

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SUMMARY

Two species and one variety of *Laetiporus* have been hitherto reported from Japan. *Laetiporus sulphureus* var. *sulphureus* auct Japan has a lemon yellow pore layer and yellow pileus surface, though it is distinct from the European form by its non-imbricated pilei and its southern distribution. *Laetiporus sulphureus* var. *miniatus* auct Japan has a white or a lemon yellow pore layer, pinkish orange pileus surface, and imbricated pilei. *Laetiporus versisporus* has semi-globose basidiocarps with abundant chlamydospores in the context when matured and usually does not produce hymenophores.

In order to define the intra-generic taxa of Japanese and European *Laetiporus* spp., 38 Japanese isolates of *Laetiporus* spp. and five European isolates of *L. sulphureus* were analyzed for variation in the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. Phylogenic analysis of the ITS region sequences resulted in five groups: Group A) the Japanese isolates of *L. sulphureus* var. *miniatus* associated with hardwoods, which has a white pore layer, Group B) the European *L. sulphureus* isolates, Group C) the Japanese isolates of *L. sulphureus* var. *miniatus* associated with conifers, which has a lemon yellow pore layer, and Groups D) and E) the Japanese isolates of both *L. sulphureus* var. *sulphureus* and *L. versisporus*. These results suggest that 1) all the Japanese *Laetiporus* spp. are distinct from European *L. sulphureus*, 2) Japanese *L. sulphureus* var. *miniatus* in association with different host types (hardwoods vs. conifers) belong to distinct taxa, and 3) Japanese *L. sulphureus* var. *sulphureus* and *L. versisporus* belong to the same species with different morphology.

Keywords: Internal transcribed spacer

INTRODUCTION

Laetiporus spp. occur worldwide from boreal to tropical zones and cause red-brown cubical heart-rot in the wood of many deciduous and coniferous trees. Recently, *Laetiporus sulphureus* (Fr.) Murr. sensu lato in North America has been shown to be a complex of at least four taxa based on incompatibility tests, restriction fragment length polymorphisms in the ITS region of the nuclear ribosomal DNA, macro morphological characteristics, geographical distribution, and host range (Banik and Burdsall 1999, 2000; Banik et al. 1998). In Europe, phylogenetic analyses based on sequences of ITS region of the nuclear ribosomal DNA indicate that *L. sulphureus* may be separated into at least two taxa in association with diferent host types (Rogers et al. 1999).

In Japan, two species and one variety of *Laetiporus* spp. have been hitherto reported. *Laetiporus sulphureus* var. *sulphureus* auct Japan has a lemon yellow pore layer and yellow pileus surface, though it is distinct from the European form by its non-imbricated pilei and its southern distribution. *Laetiporus sulphureus* var. *miniatus* auct Japan has a white or a lemon yellow pore layer, pinkish orange pileus surface, and imbricated pilei. This fungus is mainly distributed in cool temperate to boreal areas of Japan. *Laetiporus versisporus* has semi-globose basidiocarps with abundant chlamydospores in the context when matured and usually does not produce hymenophores. Its basidiocarps are at first lemon yellow then turn white to brown.

The objective of this study was to define the intra-generic taxa of *Laetiporus* spp. from Japan and Europe and based on sequence of ITS regions of the nuclear ribosomal DNA.

MATERIALS AND METHODS

Thirty-eight isolates of *Laetiporus* spp. collected throughout Japan, five isolates of European *L. sulpureus*, and two isolates of *L. porutentosus* were used in this study (Table 1).

Table 1. Laetiporus isolates used in this study.

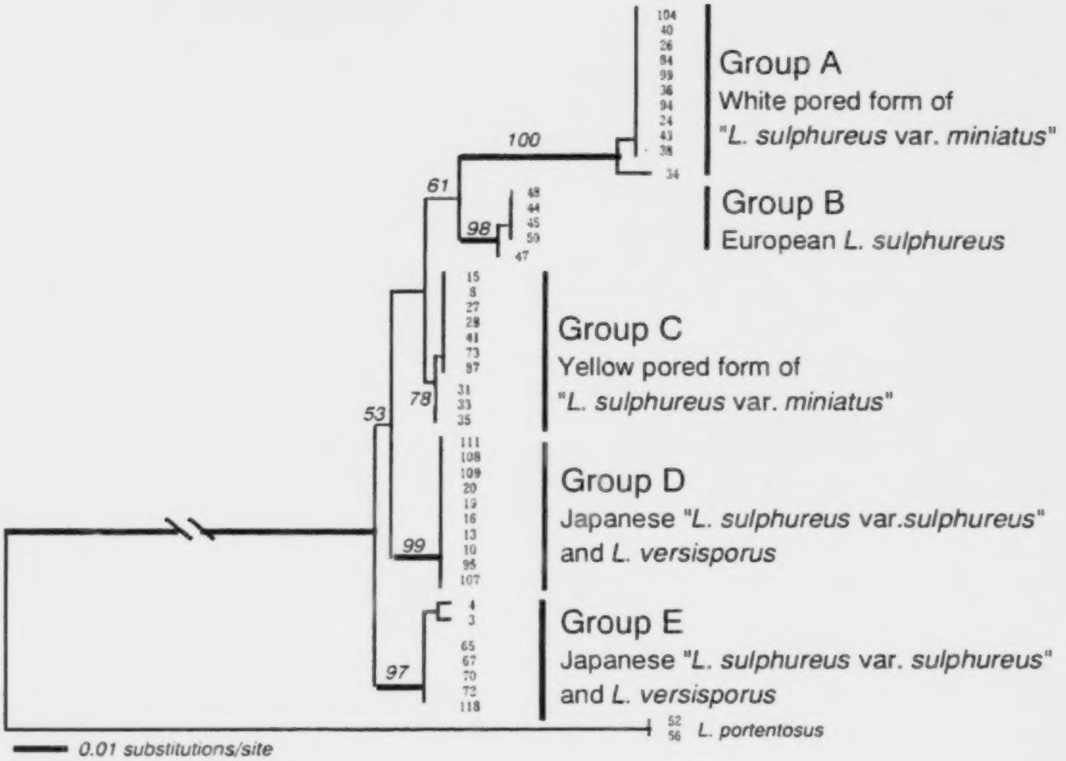
Isolate no.	Species	Host	Origin	Source
10	*L. sulphureus* var. *sulphureus*		Kouchi, Japan	
16	*L. sulphureus* var. *sulphureus*		Yamaguchi, Japan	
20	*L. sulphureus* var. *sulphureus*		Ehime, Japan	
95	*L. sulphureus* var. *sulphureus*	*Quercus* sp.	Kumamoto, Japan	
107	*L. sulphureus* var. *sulphureus*	Hardwood	Miyazaki, Japan	
108	*L. sulphureus* var. *sulphureus*		Miyazaki, Japan	
111	*L. sulphureus* var. *sulphureus*	Hardwood	Miyazaki, Japan	
118	*L. sulphureus* var. *sulphureus*	*Castanopsis cuspidata*	Kyoto, Japan	
3	*L. versisporus*	*Castanopsis cuspidata* var. Sieboldii	Tokyo, Japan	
4	*L. versisporus*	*Castanopsis cuspidata* var. Sieboldii	Chiba, Japan	
13	*L. versisporus*		Kagoshima, Japan	
19	*L. versisporus*		Mt. Baishanzu, China	
65	*L. versisporus*		Nata, Japan	
67	*L. versisporus*		Osaka, Japan	
70	*L. versisporus*	*Castanopsis cuspidata*	Kyoto, Japan	
72	*L. versisporus*	*Castanopsis cuspidata*	Kyoto, Japan	
109	*L. versisporus*	Hardwood	Miyazaki, Japan	
24	*L. sulphureus* var. *miniatus*		Hokkaido, Japan	
26	*L. sulphureus* var. *miniatus*		Yamanashi, Japan	
34	*L. sulphureus* var. *miniatus*	*Prunus Mume*	Hokkaido, Japan	T. Yamaguchi
36	*L. sulphureus* var. *miniatus*	*Prunus Sargentii*	Hokkaido, Japan	T. Yamaguchi
38	*L. sulphureus* var. *miniatus*	*Quercus mongolica*	Hokkaido, Japan	T. Yamaguchi
40	*L. sulphureus* var. *miniatus*	*Prunus salicina*	Hokkaido, Japan	T. Yamaguchi
84	*L. sulphureus* var. *miniatus*	*Quercus mongolica*	Shizuoka, Japan	
94	*L. sulphureus* var. *miniatus*	*Quercus* sp.	Miyazaki, Japan	
99	*L. sulphureus* var. *miniatus*		Ehime, Japan	
104	*L. sulphureus* var. *miniatus*		Kumamoto, Japan	
8	*L. sulphureus* var. *miniatus*	*Abies firma*	Chiba, Japan	
15	*L. sulphureus* var. *miniatus*		Yamanashi, Japan	
27	*L. sulphureus* var. *miniatus*		Yamanashi, Japan	
28	*L. sulphureus* var. *miniatus*		Aomori, Japan	
31	*L. sulphureus* var. *miniatus*	*Picea Glehnii*	Hokkaido, Japan	T. Yamaguchi
33	*L. sulphureus* var. *miniatus*	*Picea Glehnii*	Hokkaido, Japan	T. Yamaguchi
35	*L. sulphureus* var. *miniatus*	*Picea Glehnii*	Hokkaido, Japan	T. Yamaguchi
41	*L. sulphureus* var. *miniatus*	*Abies sachalinensisi*	Hokkaido, Japan	T. Yamaguchi
73	*L. sulphureus* var. *miniatus*	*Abies* sp.	Yamanashi, Japan	
87	*L. sulphureus* var. *miniatus*		Yamanashi, Japan	
44	*L. sulphureus*	*Taxus baccata*	Belgium	C. Decock
45	*L. sulphureus*	*Prunus* sp.	Belgium	C. Decock
47	*L. sulphureus*	*Malus sylvestris*	Belgium	C. Decock
48	*L. sulphureus*	Hardwood	Germany	C. Decock
50	*L. sulphureus*	*Salix* sp.	Belgium	C. Decock
52	*L. portentosus*	*Nothofagus pumilio*	Argentina	M. Rajchenberg
56	*L. portentosus*	*Eucaryptus macrororrhyncha*	Argentina	M. Rajchenberg

DNA was extracted using a CTAB (cetyltrimetylammorium bromide) procedure. The ITS regions of nuclear rDNA were amplified with PCR using primer ITS4 and ITS5 (White et al. 1990). PCR amplification was performed using Perkin-Elmer GeneAmp PCR Reagent Kit with Taq DNA Polymerase (TaKaRa, Japan). The thermal program was an initial denaturing at 92°C for 5 min, followed by 40 cycles at 55°C for 1 min (annealing), 72°C for 2 min (elongation) and 92°C for 1 min (denaturing). A final elongation was allowed for 5 min at 72°C. Sequences were determined in both directions with the same primers using a Perkin Elmer Applied Biosystems (Foster City, CA, U.S.A.) PRISM Ready Reaction DyeDeoxy Termitator Cycle Squencing kit and a Perkin Elmer Applied Biosystems Automated DNA Sequencer 310.

Sequences were aligned using Clustal X (Jeanmougin et al. 1998) and adjusted manually. Phylogenetic analysis of the aligned sequences was performed using both distance and parsimony methods. For distance analysis, the neighbor-joining method generated from HKY85 distances using PAUP 4.0b (Swofford 2001) was performed. The strength of the internal branches of the resulting trees was statistically tested by bootstrap analysis (Felsenstein 1985) from 1000 bootstrap replications. For parsimony analysis, the maximum-parsimony analysis was performed using PAUP 4.0b. 100 replicate heuristic searches were performed, each with a random taxon addion sequence, and TBR blanch swapping. These trees were rooted with the sequence of ITS of *L. portentosus* as out-group.

RESULTS

The ITS1, ITS2 and 5.8S gene were completely sequenced in both directions. There was sequence variation in the ITS sequences of *Laetiporus* isolates. By contrast, there was little variation in the sequences of the 5.8S gene. The nucleotide sequence data set obtained from the isolates in Table 1 gave a 591-nucleotide aligned sequence, including some indels due to the variable nucleotide sequence of the ITS region. After excluding the indels, 513 aligned sites remained, of which 152 sites were variable. Fig. 1 shows a neighbor-joining tree. *Laetiporus* isolates used in this study were divided into five distinct groups; Group A) the Japanese isolates of *L. sulphureus* var. *miniatus* associated with hardwoods, which has a white pore layer, Group B) the European *L. sulphureus* isolates, Group C) the Japanese isolates of *L. sulphureus* var. *miniatus* associated with conifers, which has a lemon yellow pore layer, and Groups D) and E) the Japanese isolates of both *L. sulphureus* var. *sulphureus* and *L. versisporus*. Groups A, B, D and E were strongly supported by the bootstrap analysis (almost 100%), the bootstrap value of the group C was 78%. A similar tree topology was obtained by the maximum-parsimony method using PAUP4.0b (data not shown).


104
40
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27
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87
31
33
35
78
53
111
108
109
20
19
16
13
10
95
107
99
4
3
65
67
70
72
118
97
52
56
L. portentosus
0.01 substitutions/site
Group A
White pored form of
"L. sulphureus var. miniatus"
Group B
European L. sulphureus
Group C
Yellow pored form of
"L. sulphureus var. miniatus"
Group D
Japanese "L. sulphureus var.sulphureus"
and L. versisporus
Group E
Japanese "L. sulphureus var. sulphureus"
and L. versisporus

Fig. 1. Neighbor-joining tree on distances derived from sequences of the ITS1, ITS2 and 5.8S rRNA gene of *Laetiporus* spp. Distances were determined by HKY85 methods. Bootstrap values based on 1000 replications are placed by the nodes.

DISCUSSION

Phylograms for the ITS region separated *Laetiporus* isolates into five distinct groups. European form of *L. sulphureus* (Group B) was separated from all other Japanese *Laetiporus* spp. Therefore, Japanese *Laetiporus* spp. are distinguished from European *L. sulphureus*.

Laetiporus sulphureus var. *miniatus* auct Japan was phylogenetically divided into two groups (A and C) in this study. Distinctive differences in morphological and ecological characteristics occurred between groups A and C. Group A consists of those with white pores occurring on living and dead trunks or logs and stumps of hardwoods. Group C includes the yellow pored form, which is mainly distributed in cool temperate to boreal areas in association with conifers. This group was considered to be identical with one of North American *Laeriporus* group (LIG III), which occur on conifers, fruiting on stumps and living and dead trunks and with a yellow pore layer (Banik and Burdsall 2000).

Groups D and E include both *L. sulphureus* var. *sulphureus* auct Japan, and *L. versisporus*. *Laetiporus versisporus* is different from normal *Laetiporus* spp. by their abundant chlamydospores in the context when matured, and lack of hymenophores. However, the intermediate form of *L. versisporus* and *L. sulphureus* var. *sulphureus* auct. Japan has been collected from mainly southern part of Japan. *Laetiporus versisporus* is considered to be the ptychogasteric stage of *L. sulphureus* var. *sulphureus* auct Japan. There appears to be significant geographical differentiation between Groups D and E. The isolates belonging to Group E were collected from central part of Japan. The isolates belonging to Group D were collected from southern part of Japan.

Morphological study and pairing tests to determine the compatibility of these *Laetiporus* spp. in Japan are currently underway.

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STUDIES IN *POLYPORUS* SUBG. *POLYPORELLUS*: ON CONGRUENCE OF THREE BIOLOGICAL, MORPHOLOGICAL AND PHYLOGENETIC SPECIES

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Dedicated to the victims of the brutal attack on the civilized world, Sept. 11, 2001. This paper appears in place of an invited oral presentation cancelled as a result of limitations on international air travel.

SUMMARY

The white-rotting polypore genus *Polyporus* comprises several infrageneric groups or subgenera, including *Polyporellus*, which in turn contain several similar morphological species. Crosses using monokaryotic single-spore isolates of *Polyporus arcularius*, *P. brumalis*, and *P. ciliatus* obtained from different geographic locations failed to uncover cryptic biological species. ITS rDNA sequences provided additional information on the phylogeny of these closely related members of *Polyporellus*.

Keywords: Basidiomycotina, compatibility, molecular phylogeny, ribosomal DNA

INTRODUCTION

The group *Polyporellus* contains, among a few other species, three morphotaxa widespread in the Northern Hemisphere: *P. arcularius*, *P. brumalis*, and *P. ciliatus* (Nuñez and Ryvarden 1995). *P. arcularius* is nearly cosmopolitan, but does not extend into the boreal realm. For example, in Germany there is a northern limit reported by Conrad et al. (1995). *P. brumalis* is circumhemispherical in Eurasia and North America, and *P. ciliatus* has been reported from temperate Eurasia. All three species have also been reported from South America (Popoff and Wright 1998 for *P. arcularius* and *P. ciliatus*, Nuñez and Ryvarden 1995: by virtue of accepting synonyms to *P. brumalis* and *P. ciliatus* from Spegazzini). Closely related to *P. ciliatus* appears to be *P. tricholoma*, included in our phylogenetic analyses for outgroup purposes.

Spanish and Costa Rican collections of *P. arcularius* have previously been shown to belong to the same intercompatibility group (Nuñez 1993). Likewise, Hoffmann (1978) reported collections of *P. ciliatus* from Europe and North America to belong to one interfertile group and collections of *P. brumalis* from Europe, North America, and India to form another interfertile group. There has been nomenclatural confusion in *P. arcularius*, *P. brumalis*, and *P. ciliatus*, and names have been misapplied (see Jahn 1969; Kreisel 1963). Based on mating study results, Hoffmann (1978) assigned two German and one Canadian collection obtained as *P. brumalis* to *P. ciliatus*. For Hoffmann (1978) all three taxa (with one strain of *P. arcularius* included) were mutually incompatible.

A particular question is whether *Polyporus ciliatus* occurs in North America, as claimed by Hoffmann (1978) and herbarium labels at DAOM, or in South America, as indicated by Popoff and Wright (1998). Nuñez and Ryvarden (1995) regarded *P. ciliatus* as unknown from North America. We are also interested in addressing the phylogeny of *Polyporellus*, and whether different morphospecies correspond to single biological and phylogenetic species entities.

Here we report current progress of our research in *Polyporellus* as we collect the initial framework of data to answer the above problems, including an assessment of the suitability of the use of nuclear ITS rDNA data for the questions of interest.

MATERIALS AND METHODS

Specimens, establishment and maintenance of cultures

Our own collections were assigned field book numbers, annotated, dried for preservation, and accessioned into TENN (Holmgren et al. 1981). Phase contrast microscopic observations were undertaken after squash mounting of herbarium specimens in 3% w/v KOH and phloxin dye, at 400 x magnification. Identification based on morphological characters was accomplished with the aid of keys by Gilbertson and Ryvarden (1986 - 1987), Ryvarden and Gilbertson (1993 - 1994), and Nuñez and Ryvarden (1995). In some cases, colleagues furnished spore prints from which cultures were obtained.

Techniques for establishing monokaryotic single-basidiospore isolates (SBIs) were described by Gordon and Petersen (1991). Dikaryon cultures were established for a number of collections as described by Petersen and Hughes (1997). Monokaryon and dikaryon cultures were stored on agar disks of malt extract agar (MEA: 1.5% w/v Difco malt extract, 2% w/v Difco Bacto-agar, Nobles 1965) in sterile water in microvials (Burdsall AND Dorworth 1994). Occasional bacterial contamination was overcome by passage through MEA plates supplemented with 1.54 mM chloramphenicol (Calbiochem 220551) and 10.67 µM streptomycin sulfate (Sigma S0890).

In order to obtain lacking herbarium specimens and/or monokaryon cultures, fruiting of dikaryon cultures on *Fagus*, *Acer*, *Juglans*, or *Betula* wood chips followed the procedure of Psurtseva and Mnoukhina (1998). Additional dikaryotic cultures were obtained from culture collections, and specimens or DNA extractions from other sources below. The following section, ordered alphabetically by country of origin, is read as: COUNTRY. Infracountry. Notes. *Collector* and *date*. **Field book number** or other number obtained elsewhere / **TENN number** or other collection number if available **(GenBank number)**. Other acronyms: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands. DAOM = "Department of Agriculture, Ottawa, Mycology" (National Mycological Herbarium, Ottawa, Canada). DSH = D. S. Hibbett collection number. DSMZ-H = cultures used by Hoffmann (1978) and kept at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. O = University of Oslo herbarium, Oslo, Norway. SBUG-M = "Sektion Biologie Univ. Greifswald - Myzelpilze" (Univ. Greifswald, Germany, fungal culture collection). VT = "Virginia Tech".

***P. arcularius* Batsch: Fr.**

AUSTRIA. Niederösterreich: Kaltenleutgeben. On *Fagus*. *H. Voglmayr Apr 04 1999*. **10299/TENN58370 (**SBI2: **AB070865**, SBI4: **AB070866)**. / CANADA. Ontario: Rondeau Prov. Park. On *Tilia*. *R. G. Thorn May 22 1983*. **CBS 223.91/ RGT830522/01 (AB070858)**. / COSTA RICA. Cartago: km 66 of Interamerican Highway. *R. Petersen Jul 01 1998*. **9473/TENN56447**. / CHINA. Guizhou. *R. Petersen Aug 31 1991*. **4124/TENN50834** (SBI1: **AB070863**, SBI2: **AB070864)**. / GERMANY. Mecklenburg-Vorpommern: Usedom. On *Fagus*. *R. Bütow Jun 1997*. **SBUG-M1244** (fruited for obtaining specimen/spores) **/TENN58529, 58569, 58588 (AB070861)**. / SOUTH AFRICA. Kruisfountein. On *Olea*. *P. Talbot Nov 1955*. **DAOM 94067 (from PRE52) (AB070859)**. / USA. Florida: Welaka. On wood chips. *E. Lickey Mar 19 2001*. **10975/TENN58890**. / USA. Louisiana: Baton Rouge. *D. Sime May 21 1997*. **9076/TENN54876**. / USA. Louisiana: Lafayette. *R. Petersen May 23 1997*. **9101/TENN54925**. / USA. Louisiana: West Feliciana. *E. Lickey May 26 1997*. **9214/TENN55948**. / USA. Tennessee: Tremont. *H. Voglmayr Apr 02 2000*. **10929/TENN58412 (**SBI1: **AB070867**, SBI2: **AB070868)**. / USA. Tennessee: Knoxville. *H. Voglmayr Apr 04 2000*. **10930/TENN58438**. / USA. Texas. On *Pinus*. *J. L. Mata Jun 10 2000*. **10477/TENN58540**. / USA. Texas. *A. Methven Jun 10 2000*. **10693/TENN58779**. / Extraction from D. S. Hibbett (Hibbett and Donoghue 1995). **DSH92.144 (AB070860)**. / Extraction from D. S. Hibbett (Hibbett and Vilgalys 1993). **VT959 (AB070862)**.

***P. brumalis* Pers.: Fr.**

AUSTRIA. *I. Krisai-Greilhuber Sep 28 1996.* **8037/TENN55596**. / AUSTRIA. *I. Krisai-Greilhuber Jun 10 1996.* **8038/TENN55597**. / AUSTRIA. Niederösterreich: Muckendorf. *I. Krisai-Greilhuber and H. Voglmayr Oct 04 1998.* **10123/TENN57347**. / AUSTRIA. Oberösterreich: St. Willibald. On *Betula*. *H. Voglmayr Oct 23 1999.* **10666/TENN58382**. / AUSTRIA. Oberösterreich: Kirchschlag. On *Sorbus*. *H. Voglmayr Nov 01 1999.* **10667/TENN58383**. / CANADA. Quebec: Kingsmere. *J. W. Groves Oct 31 1955.* **DAOM 31983 (AB070869)**. / CANADA. Quebec: Kingsmere. *J. W. Groves Oct 31 1955.* **DSMZ-H17 (from DAOM 31983) (AB070870)**. / DENMARK. Roskilde Amt: Lellinge. On *Fagus*. *H. Knudsen May 18 1999.* **10169/TENN57700** (SBI1: **AB070872**, SBI3: **AB070873)**. / DENMARK. Storstrøms Amt: Fakse. On *Fraxinus*. *R. Petersen May 19 1999.* **10178/TENN57708**. / GERMANY. Mecklenburg-Vorpommern: Malchow. On *Fagus*. *D. Krüger May 09 1999.* **10147/TENN57678**. / GERMANY. Mecklenburg-Vorpommern: Neustrelitz. On *Fagus*. *D. Krüger Dec 28 1999.* **10908/TENN58391** (SBI4: **AB070876**, SBI5: **AB070877)**. / NORWAY. Oslo. On *Betula*. *D. Krüger Mar 25 2000.* **10917/TENN58400**. / NORWAY. Telemark: Grasdalen. On *Sorbus*. *A.-E. Torkelsen May 23 1972.* **O92301 (AB070871)**. / RUSSIA. On *Alnus*. *R. Petersen Sep 21 1996.* **8971/TENN55631**. / USA. Alaska: Anchorage. On *Alnus*. *R. Petersen Sep 09 1995.* **7992/TENN53984** (was identified as *P. ciliatus*). / USA. Alaska: Anchorage. On *Alnus*. *R. Petersen Sep 11 1995.* **8122/TENN53936** (was identified as *P. ciliatus*). / USA. Tennessee. On *Fagus*. *K. McFarland Nov 07 1999.* **10665/TENN58381** (SBI1: **AB070874**, SBI2: **AB070875)**. / USA. West Virginia. *A. Kovalenko Sep 30 2000.* **10964/TENN58828**. / USA. West Virginia. *A. Kovalenko Sep 30 2000.* **10965/TENN58827**.

***P. ciliatus* Fr.**

AUSTRIA. Niederösterreich: Hainburg. On *Populus*. *I. Krisai-Greilhuber and H. Voglmayr Apr 29 1999.* **10300/TENN58371**. / DENMARK. Ribe Amt: Billund. *J. Vesterholl May 14 1999.* **10507/TENN57737**. / DENMARK. Roskilde Amt: Lellinge. *R. Petersen, H. Knudsen and D. Krüger May 18 1999.* **10165/TENN57696**. / DENMARK. Roskilde Amt: Lellinge. On *Acer*. *H. Knudsen and R. Petersen May 18 1999.* **10167/TENN57698** (SBI9: **AB070882**, SBI10: **AB070883)**. / DENMARK. Roskilde Amt: Lellinge. On *Fagus*. *H. Knudsen and D. Krüger May 18 1999.* **10168/TENN57699**. / GERMANY. Baden-Württemberg: Tübingen. On *Betula*. *D. Krüger May 31 1999.* **10521/TENN57751**. / GERMANY. Mecklenburg-Vorpommern: Malchow. On *Quercus*. *D. Krüger May 05 1999.* **10149/TENN57680**. / GERMANY. Mecklenburg-Vorpommern: Zislow. On buried *Betula* wood. *D. Krüger May 05 1999.* **10151/TENN57682**. / GERMANY. Mecklenburg-Vorpommern: Neustrelitz. On *Fagus*. *D. Krüger May 13 1999.* **10156/TENN57687**. / GERMANY. Mecklenburg-Vorpommern: Greifswald. On *Fagus*. *M. Scholler and D. Krüger May 22 1999.* **10181/TENN57711**. / GERMANY. Mecklenburg-Vorpommern: Carpin, Nature Reserve Serrahn. *R. Petersen May 25 1999.* **10318/TENN57966**. / GERMANY. Mecklenburg-Vorpommern: Zwenzow, Krummer See. *D. Krüger May 26 1999.* **10320/TENN57968**. / GERMANY. Mecklenburg-Vorpommern: Carpin, Nature Reserve Serrahn. On *Fagus*. *D. Krüger May 25 1999.* **10508/TENN57738**. / FINLAND. Etelä-Häme: Evo. *R. Petersen Sep 13 1994.* **7480** (fruited for obtaining spores to make up for lost monokaryons) **/TENN53639, 58441, 58823** (SBI2: **AB070880**, SBI3: **AB070881)**. / SWEDEN. Uppland: Tarnby Lund. On *Betula*. *R. Petersen Sep 04 1994.* **7257/TENN53619** (SBI2: **AB070878**, SBI7: **AB070879)**.

***P. tricholoma* Mont. (included in phylogeny)**

COSTA RICA. Heredia: Chilimate. On buried wood. *R. Petersen Mar 13 1999.* **10240/TENN57563 (AB070885)**. / COSTA RICA. Heredia: Chilimate. *R. Petersen Mar 13 1999.* **10241/TENN57564 (AB070888)**. / MEXICO. Chiapas. Small dead angiosperm branchlets. *R. Petersen Oct 18 1997.* **3870/TENN55844 (AB070884)**. / USA. Puerto Rico: Palmer. Hardwood log. *R. Petersen Jun 09 1998.* **9568/TENN56481 (AB070887)**. / USA. Puerto Rico: Palmer. Hardwood log. *R. Petersen Jun 12 1998.* **9591/TENN56503 (AB070886)**.

P. alveolaris (DC.: Fr.) Bondartsev and Singer (*Favolus* group; outgroup in phylogeny)
Extraction from D. S. Hibbett (Hibbett and Vilgalys 1993). **DSH 90.36 (AB070828)**.

Mating experiments

Self-cross pairings for determination of mating types and tester strains were made among 12 randomly selected monokaryotic SBIs of at least one collection per species. Tester strains are SBIs with known mating type assigned for later testing of new arrivals. Subtester strains are auxiliary tester monokaryons selected later from collections of a different geographic origin, without prior knowledge of the actual mating type of the SBI. The technique for self-crosses was described by Petersen (1992). For intercollection pairings, four SBIs (randomly selected, or testers/subtesters when assigned) were paired with four SBIs (randomly selected, or testers/subtesters when assigned) from other collections in this study.

DNA extraction

DNA was extracted using a CTAB method (either modified from Carlson et al. 1991, see Hughes et al. 1999; or modified from Doyle and Doyle 1987; Zolan and Pukkila 1986; see Krüger et al. 2001), SDS-based method (Lee and Taylor 1990), or more recently, with a xanthogenate/SDS miniprep protocol modified from Tillett and Neilan (2000). All methods gave approximately equivalent results and were not always effective. In the CTAB methods, 10 to 50 mg of herbarium material, or less of hyphal material scraped off MEA plates, was placed in a 1.5 ml reaction tube with 0.5 ml prewarmed (65°C) CTAB extraction buffer (0.1M Tris, 0.2 M Na_2EDTA, 1.5 M NaCl, 55 mM CTAB = hexadecyltrimethylammonium bromide, Sigma H5882). The material was incubated for 30 to 60 min at 65°C in a 1.5 ml tube and shaken occasionally before being ground with a sterilized plastic mini pestle (Kontes Pellet Pestle®, Kontes 749520).

For tough basidiocarps of herbarium specimens, grinding was supported with sterile sand, and the material repeatedly frozen and heated (three cycles of 10 min at -80°C / 10 min at 65°C (Vrålstad et al. 2000). Here, a 3% w/v SDS (sodium dodecyl sulfate, Sigma L4509), 1% w/v mercaptoethanol extraction buffer (500 µl, 65°C) was used instead of CTAB extraction buffer.

With either extraction procedure, approximately the same volume of 24:1 chloroform:isoamyl alcohol was added, the mixture vortexed briefly, and spun at 12,000 rpm for 4 min. The upper, clear phase was transferred to a new 1.5 ml reaction tube and mixed with an equal volume of isopropyl alcohol (4°C). The reaction tube contents were mixed, refrigerated (4°C) for one hour, and centrifuged for 4 min at 13,000 rpm. The resulting pellet was rinsed twice in 70% v/v ethyl alcohol, air-dried, and resuspended in 100 µl TE buffer (10 mM TrisHCl, 1 mM Na_2EDTA; pH 8.0).

DNAs were also extracted from fungal tissue using a modified xanthogenate protocol (Tillett and Neilan 2000). Fresh material was soaked for several weeks at 4°C in CTAB / sodium azide preparations (after Rogstad 1992: 6 M NaCl, 3 mM NaN_3, 41.1 mM CTAB). Alternatively, fresh or herbarium material was stored in SDS buffer (50 mM Tris/HCl, 50 mM Na_2EDTA, 10% w/v SDS, pH 7.2). Ten to 50 mg of such material was ground in 50 µl TE buffer with a small amount of sterile sand in a 1.5 ml microfuge tube as described above. For material grown 2 - 8 weeks at room temperature in malt extract (ME) broth (ca. 10 ml 1.5% w/v Difco malt extract, in baby food jar), 250 - 500 mg of material was filtered, blotted dry, then ground. After addition of 50 µl TE extraction buffer, a minipestle mounted on a drill was used to grind the material. Following grinding, 750 µl of potassium ethyl xanthogenate buffer (100 mM Tris/HCl pH 7.2, 20 mM Na_2EDTA pH 8.0, 1% w/v SDS, 800 mM ammonium acetate, 1% w/v $C_3H_5KOS_2$ = potassium ethyl xanthogenate: Fluka 60045) was added. The tube contents were vortexed, and incubated at 70°C for 60 min, with occasional vortexing. After a final, vigorous vortex for 10 sec, samples were placed on ice for 30 - 60 min, and then centrifuged at 14,000 rpm for 10 min. The supernatant was recovered, and DNA precipitated with 750 µl isopropyl alcohol (80% v/v, 4°C), and spun at 10,000 rpm for 10 min. The alcohol was aspirated, and the pellet was washed with 250 µl 95% v/v cold ethyl alcohol, spun again at 10,000 rpm for 10 min. Remaining alcohol was then pipetted off, removed with a piece of paper towel, and evaporated 2 min at 70 °C on a heating block. The pellet was then resuspended in 50 µl TE buffer supplemented with 2 µl RNAse Plus (5 Prime - 3 Prime, Inc., now Eppendorf-5 Prime, Inc.), and incubated 10 min at 50°C.

PCR and sequencing

DNA selected for sequencing was primarily monokaryotic in origin. For tester and subtester isolates we attempted to sequence two monokaryons for each collection. The nuclear ribosomal ITS I - 5.8S - ITS2 region was amplified with primers ITS 1-F and ITS 4-B (Gardes and Bruns 1993). A 50 µl reaction contained 1 X buffer supplied by manufacturer (includes $MgCl_2$ in case of QBioGene polymerase kit, or separate 3 mM $MgCl_2$), 0.8 mM each dNTP, 0.2 ng/µl of bovine serum albumin (Sigma A7906), 0.2 mM primer each, 1.1 units *Taq* Polymerase (QBioGene EPTQA023; for difficult reactions: TaKaRa ExTaq ™ kit used after manufacturer's instructions, PanVera). Parameters were as follows: initial denaturation 94°C / 3 min, followed by 35 cycles of denaturation 94°C / 1 min, annealing 52°C / 1 min, extension 72°C / 1 min. The final extension was 72°C / 3 min, followed by an indefinite storage step at 4°C. PCR products were electrophoresed in a 1.5% w/v agarose/TBE gel. In spite of secondary bands, amplified DNA product was not excised, but used after cleaning with a Microcon-PCR device kit (Millipore), according to manufacturer's instructions. This seemed appropriate because disappearance after treatment with restriction enzymes *Bfi*I (MBI Fermentas) and *Nci*I (New England Biolabs) in *Polyporus tricholoma* proved those bands to be due to secondary structure. Internal primers ITS 5 and ITS 3 (White et al. 1990) were used as sequencing primers. Cycle-sequencing was performed with the ABI Prism Dye Terminator Cycle Sequencing kit (Perkin-Elmer), following manufacturer's instructions and using approximately 200 ng DNA template.

Sequence data

Sequences were corrected using Chromas v. 1.45 (Conor McCarthy; Griffith University, Southport, Australia) for viewing and manipulating ABI electropherograms and Programmer's File Editor v. 0.07.002 (Alan Phillips, Lancaster University, UK). Sequence alignment was done with ClustalX 1.64b (Thompson et al. 1997), followed by visual confirmation and manual optimization. The neighbor-joining (Saitou and Nei 1987) algorithm as implemented in ClustalX was used for initial phylogenetic analysis. A NEXUS file (Maddison et al. 1997) was manually generated for use in PAUP*4.0b8 (Swofford 2001). Gaps were coded as missing after trying alternative treatment modes. Heuristic searches were performed both in 20% deletion jackknife (Efron and Gong 1983) and bootstrap (Felsenstein 1985) resampling analyses (TBR swapping, MAXTREES set to 1,000, 100 random taxon addition repeats per resampling pseudoreplicate). TreeVIEW 1.5 (Page 1996) was used to view and manipulate trees. Sequences were submitted to EMBL/GenBank/DDBJ databases using the DDBJ Sakura submission system.

RESULTS

Mating studies and culture morphology

We confirmed a tetrapolar mating system for *P. arcularius* (Vandendries 1936a, Hoffmann 1978), *P. brumalis* (Vandendries 1936b, Hoffmann 1978) and *P. ciliatus* (Hoffmann 1978, Petersen et al. 1997). We selected the following tester strains for later research: *P. arcularius* field book number 10299: A_1B_1 (SBI1), A_1B_2 (SBI4), A_2B_1 (SBI3), A_2B_2 (SBI2); *P. brumalis* field book number 10908: A_1B_1 (SBI1), A_1B_2 (SBI4), A_2B_1 (SBI8), A_2B_2 (SBI5); and *P. ciliatus* field book number 10167: A_1B_1 (SBI11), A_1B_2 (SBI9), A_2B_1 (SBI10), A_2B_2 (SBI12). Results of the intercollection pairing experiments are shown in Fig. 1B (lower triangle).

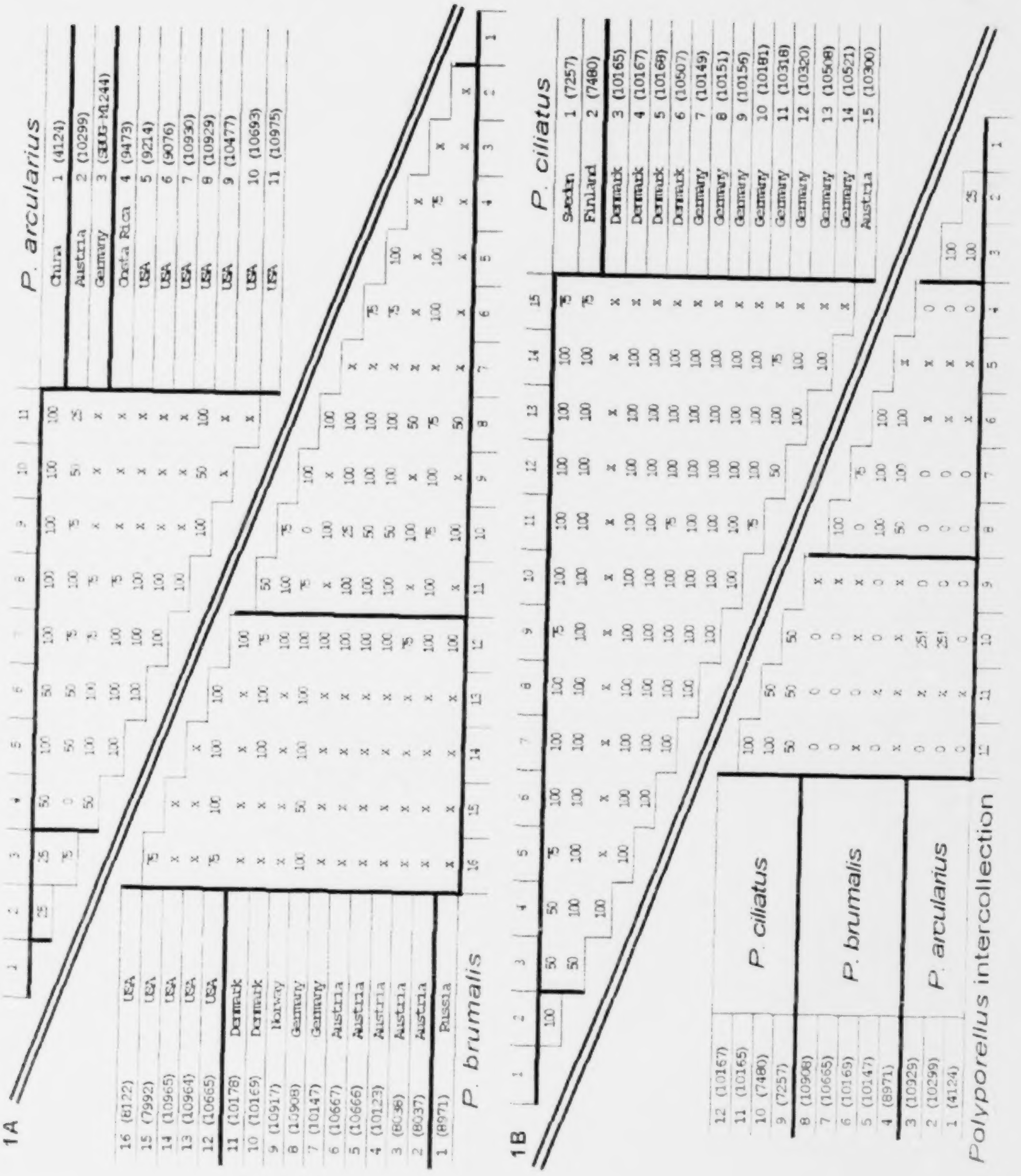
1A
P. arcularius
P. brumalis
1B
P. ciliatus
P. ciliatus
P. brumalis
P. arcularius
Polyporellus intercollection

Figure 1. Schematic overview of mating compatibility. Major geographic regions separated by broader cell boundaries. Numbers on upper and lower end of image correspond to the collections (field book numbers) left and right-hand. X = not tested. 100 = 4/4 pairings form clamps, 75 = 3/4, 50 = 2/4, 25 = 1/4, 0 = 0/4. 1A *P. arcularius* (upper triangle) and *P. brumalis* (lower triangle). 1B *P. ciliatus* (upper triangle) and cross-species within three taxa (lower triangle). ! = unexpected formation of clamps, confirmed by a repetition.

Cultures of newly isolated *Polyporellus* SBIs tended to form basidiocarp primordia on MEA plates and in ME broth (also observed by Hoffmann 1978), a phenomenon that seems to disappear after one passage through storage in water vials. Brown crusts were formed, but were less apparent than in the *Melanopus* group. Conidiogenesis occurs, but was less frequent than in the *Squamosus* and *Melanopus* groups. The "Border Zone" mentioned by Hoffmann was not consistently evaluated, and not distinguished by us from barrage-like non-self contact zones. Several SBIs of *P. arcularius* field book number 10929 and most of *P. brumalis* field book number 8971 tended to form "halos" around the inoculation block where hyphae lay appressed on the agar surface, and more aerial growth beyond the halo. "Barrage" and "flat" reactions were not useful in assigning mating types, as mentioned by Petersen et al. (1997), and as explained by the control through mating-type independent genes *bfI*, *bfII*, and *bi* (Hoffmann 1978). Several older SBIs of *P. ciliatus* (used in Petersen et al. 1997) appeared to have lost viability in the course of repetitive work with them over several years (few aerial hyphae, only thin repent hyphae on wetted agar surface), resulting in replacement of tester stocks.

Molecular phylogeny

Sequence analyses were based on 610 aligned characters, of which 462 were stable, 75 variable but parsimony-uninformative, and 73 parsimony-informative. In the ITS 1, ITS 2 and 5.8 S downstream end regions, numerous ambiguous positions were found, but were left as ambiguous base codes in the analyses reported here. It is not yet known how much of the ambiguity stems from PCR artifacts or to what extent the rDNA repeat may not be completely homogenized.

Results of the neighbor-joining (NJ) function in ClustalX performed with 1,000 bootstrap repeats are given in Fig. 2. NJ bootstrap support (out of 1,000 trees, only if above 500) is given next to nodes. This dendrogram is rooted with *P. alveolaris*, and also gives the PAUP* jackknife and bootstrap supports for major nodes. Nodes not showing PAUP* heuristic support values were either not recovered at all in majority consensus of trees found by these parsimony methods, or were recovered at a percentage lower than 50. *P. brumalis* consisted of two NJ-clades (low NJ bootstrap support) in a trichotomy with *P. arcularius*, but was found as one single clade in PAUP* resampling analyses. This clade itself is collapsed, but supported with 99% jackknife value vs. 89% bootstrap value. In general, jackknife values are higher than bootstrap values. With high resampling support values, *P. arcularius*, *P. brumalis*, and *P. ciliatus* clades are consistently recovered with the different cladistic methods (NJ: bootstrap, heuristic: bootstrap, jackknife) employed.

A PAUP* bootstrap analysis with 100 pseudoreplicates was unable to perform more than one random taxon addition repeat (instead of 100 intended) in each pseudoreplicate as the buffer of 1,000 trees was reached within few sec. The 50% majority rule consensus scored with CI=0.840, HI=0.160, RI=0.890, RC=0.747. The 100 pseudoreplicates jackknife resampling analysis on PAUP*, based on 20% deletion (122 actual characters) also could not run the intended 100 random taxon addition replicates for the same reason as mentioned for the bootstrap. Its 50% majority rule dendrogram had following scores: CI=0.687, HI=0.313, RI=0.900, RC=0.767.

Initially we tried performing the analysis with GAPMODE=newstate. Here we reduced gaps longer than one character to one position. Several gap-carrying positions with unclear homology were excluded, and in one case those were treated as missing data. All these analyses converge on the same topology of above major clades in a majority rule consensus of 1,000 heuristic bootstrap analysis trees in PAUP*. Therefore APMODE=newstate was not used for time-consuming resampling analyses.

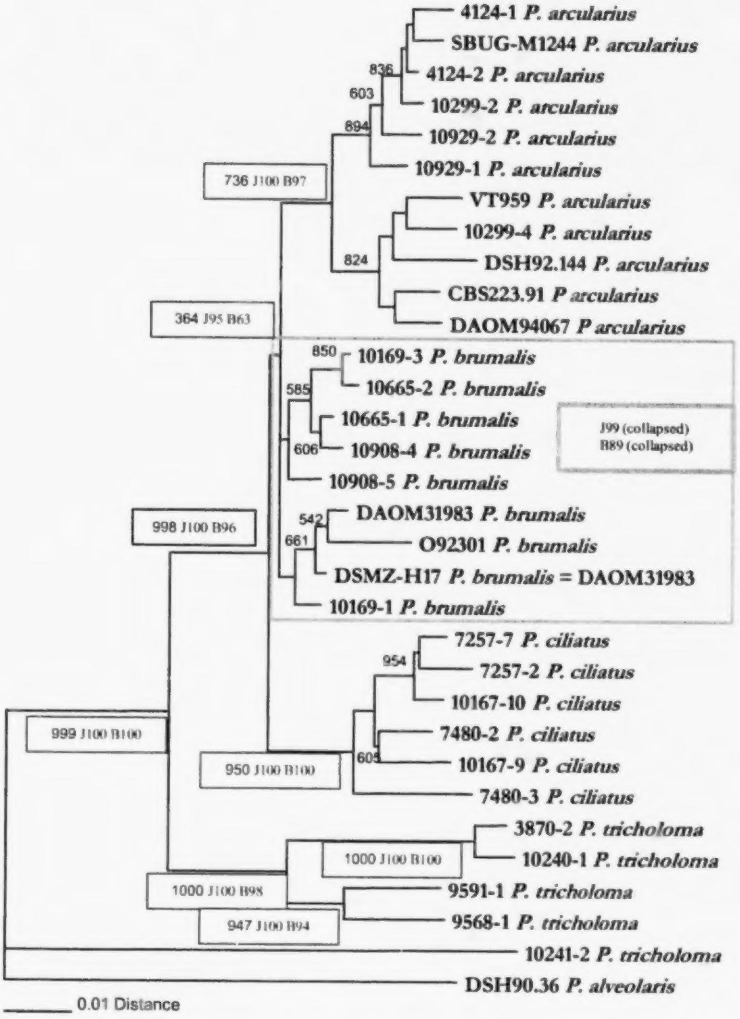
4124-1 *P. arcularius*
SBUG-M1244 *P. arcularius*
836
4124-2 *P. arcularius*
603
10299-2 *P. arcularius*
894
10929-2 *P. arcularius*
736 J100 B97
10929-1 *P. arcularius*
VT959 *P. arcularius*
10299-4 *P. arcularius*
824
DSH92.144 *P. arcularius*
CBS223.91 *P arcularius*
364 J95 B63
DAOM94067 *P arcularius*
850
10169-3 *P. brumalis*
585
10665-2 *P. brumalis*
10665-1 *P. brumalis*
J99 (collapsed)
B89 (collapsed)
606
10908-4 *P. brumalis*
10908-5 *P. brumalis*
542
DAOM31983 *P. brumalis*
998 J100 B96
661
O92301 *P. brumalis*
DSMZ-H17 *P. brumalis* = DAOM31983
10169-1 *P. brumalis*
7257-7 *P. ciliatus*
954
7257-2 *P. ciliatus*
10167-10 *P. ciliatus*
999 J100 B100
7480-2 *P. ciliatus*
605
950 J100 B100
10167-9 *P. ciliatus*
7480-3 *P. ciliatus*
3870-2 *P. tricholoma*
1000 J100 B100
10240-1 *P. tricholoma*
1000 J100 B98
9591-1 *P. tricholoma*
947 J100 B94
9568-1 *P. tricholoma*
10241-2 *P. tricholoma*
DSH90.36 *P. alveolaris*
0.01 Distance

Figure 2. ClustalX NJ dendrogram. SBI number following field book number for sequenced monokaryons in given (e.g. 9591-1: 1 denotes SBI1 of field book number 9591). NJ bootstrap support (out of 1000) is given at left of the node. Boxes at major nodes contain following values: first number: ClustalX NJ bootstrap support, second number: parsimony analysis using jackknife resampling: jackknife (J) support as a percentage, third number: parsimony analysis using bootstrap resampling: bootstrap (B) support as a percentage. Black box around *P. brumalis* indicates two NJ clades collapsing to one single, polytomous clade in PAUP* parsimony resampling analyses.

CONCLUSION

Morphology

The three morphospecies may be successfully separated using keys and descriptions by Gilbertson and Ryvarden (1986 - 1987), Ryvarden and Gilbertson (1993 - 1994), and Nuñez AND Ryvarden (1995), although we experienced some difficulty with aged basidiocarps. Where in doubt, availability of mating experiments allowed assignment to the species.

Compatibility

Polyporus arcularius / *P. brumalis* / *P. ciliatus* were each shown to have a tetrapolar mating system. Each morphospecies is congruent with one biological species by virtue of clamp formation in mate recognition. Two exceptional formations of clamps in the contact zone of plates with *P. arcularius* X *P. ciliatus* (Fig. 1B, note "!") were confirmed in a repetition; this apparent dikaryotization is awaiting further study. Aside from this, no other compatibility was detected across morphospecies boundaries.

Molecular phylogenetic reconstruction

In *P. arcularius* / *brumalis* / *ciliatus* we found that phylogenetic species confirmed the biological species entities. The major phylogenetic clades are recovered with NJ, jackknife, and bootstrap analyses. *P. arcularius* and *P. brumalis* appear to be sister taxa, with *P. ciliatus* further removed in a mid-position to ancestral *P. tricholoma*. Both *P. ciliatus* and *P. tricholoma* have smaller pores and more apparent cilia than *P. arcularius* / *brumalis*, which may mirror this relationship. We also conclude that ITS rDNA may be suitable to assign specimens / cultures to phylogenetic clades, but it is too laden with gaps, homoplasy, and ambiguity to resolve phylogeographic groups within these major clades.

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IDENTIFICATION OF *ARMILLARIA* SPP. IN NORTH-WEST SPAIN USING MOLECULAR TECHNIQUES

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SUMMARY

The genus *Armillaria* contains many species distributed worldwide. In Europe, seven different species have been identified. The presence of *Armillaria* in Spain is well known, but until now, there were no studies done on the identification and geographical distribution of this fungus. The aim of this project was to identify the different *Armillaria* species present in north-west Spain using molecular techniques.

The material used for DNA extraction was from mycelium found on affected plants, rhizomorphs found on roots, mycelium or rhizomorphs growing on agar media or from spores. Using a polymerase chain reaction (PCR) based technique, a small section of the intergenic spacer (IGS) was amplified from the material collected from different locations. A fragment of about 900 bp was amplified from all the tested samples. The amplified fragment was digested with the restriction enzymes *Alu* I, *Nde* I and *Bsm* I.

Over 100 samples have been analysed with this method and the results obtained show five different restriction patterns that corresponded to the species *A. mellea*, *A. ostoyae*, *A gallica* and *A. cepistipes*. Two different patterns were found for *A. mellea* (mel 1 pattern and mel 2 pattern). The species more frequently found was *A. mellea* (mel 2 pattern), isolated mainly from broadleaf trees, ornamental shrubs, conifers, fruit trees and vine. *A. ostoyae* has been identified only from conifers. *A. gallica* and *A. cepistipes* were less frequently found and only on broadleaf trees and vine samples.

Keywords: *Armillaria*, identification, PCR-RFLP, Spain, species

INTRODUCTION

The genus *Armillaria* contains many species distributed worldwide. In Europe, seven different species have been identified. The presence of *Armillaria* root disease in Spain is well known, but identifications of the fungus to species level are infrequent in comparison to other European countries (Guillaumin et al. 1993).

During the last ten years the number of *Armillaria* samples which have been received at the plant pathology laboratory at the Estacion Fitopatologica 'Do Areeiro' (Pontevedra, NW-Spain) has increased considerably. Amongst the plant genera examined from this region, *Vitis* was the most frequently affected. This could be related to the soil and climate conditions of the area as well as the habit of cultivating vineyards on previously wooded land.

Over a long period of time, different methods have been used to develop a rapid and accurate technique to discriminate the different *Armillaria* species. However, for various reasons, it has not been practicable to use them (Mansilla et al. 2000). After Anderson and Stasovski (1992) sequenced the IGS (Intergenic Spacer) region of the ribosomal RNA for some *Armillaria* species, a PCR- RFLP technique was developed (Harrington and Wingfield 1995) and led to the reliable identification of the different species (Pérez Sierra et al. 1999). The aim of the research reported here was to identify the different *Armillaria* species present in north-west Spain using this PCR-RFLP technique.

MATERIALS AND METHODS

Armillaria was isolated from mycelium found under the bark of infected plants, rhizomorphs found on roots, mycelium or rhizomorphs growing on agar media or from basidiospores. One hundred samples were examined from different areas in north-west Spain.

The PCR-RFLP technique used by Pérez Sierra et al. (1999) was modified by Mansilla et al. (2000) to amplify the IGS region of *Armillaria*. DNA was extracted following the protocol described for the EZNA fungal DNA miniprep kit (Omega Biotek) without RNAse or mercaptoethanol (Martin and Torres 1998). Ready-To-Go-PCR beads (Amersham-Pharmacia Biotech) were used for the amplification, adding 1µl of extracted DNA and 0.5µl of each primer (10pm/µl), in a total volume of 25µl. The primers LR12R and O-1 (Anderson and Stasovski, 1992) were selected and the amplification performed in a Mastercycler Personal thermocycler (Eppendorf). The amplification conditions were 95 seconds at 95°C, followed by 35 cycles of 60 seconds at 60°C, 120 seconds at 72°C and 60 seconds at 95°C, with a final extension of 10 minutes at 72°C.

Each time, a negative control was used as well as positive controls of haploid testers of the six European *Armillaria* species, kindly donated by Dr. Guillaumin (INRA, Clermont Ferrand, France) and Dr. Korhonen (Finnish Forest Institute Research, Finland).

The PCR products were digested with the restriction enzymes *Alu* I, *Nde* I and *Bsm* I (Roche Diagnostics) according to the manufacturer's recommendations and required purification with the High Pure PCR Product Purification kit (Roche Diagnostics) for the latter two restriction enzymes. The PCR product and the digested fragments were separated in 3% agarose gels stained with ethidium bromide. A 100 bp ladder was used as a marker (Marcador XIV, Roche Diagnostic). A gel documentation system was used to save the gel images and later these were analysed by densitometry (1D-Manager, TDI, Madrid)

RESULTS AND DISCUSSION

A fragment of about 900 bp was amplified from all the samples. The digestion of this fragment with the restriction enzyme *Alu* I gave different patterns which corresponded with the patterns of *A. mellea* (mel 1 y mel 2), *A. gallica* (gal 1) and *A. cepistipes* (cep 1) published by Pérez Sierra et al. (1999). The digestion with *Alu* I gave a restriction pattern (310, 200 and 135 bp) that corresponded with the patterns described for *A. ostoyae, A. cepistipes* 2 and *A. borealis* 2.(Harrington and Wingfield 1995; Pérez Sierra et al. 1999). The PCR products of the samples with this pattern were then digested with the restriction enzymes *Nde* I and *Bsm* I, and the samples were identified as *A. ostoyae*.

A. mellea was identified in 69% of the samples examined and the affected plants were vines, fruit trees, conifers, deciduous trees and ornamentals. The majority corresponded with *A. mellea* pattern 2 (320, 155 bp). *A. gallica* was identified in 19% of the samples and was found on vines and broadleaf trees; the digestion pattern corresponded to *A. gallica* 1 (400, 240, 190 bp). *A. cepistipes* was identified from rhizomorphs in one sample only, its restriction pattern was *A. cepistipes* 1 (400,200,190 bp). *A. ostoyae* was identified in the remainder of the samples and was always associated with *Pinus* sp. A list of the hosts and fungal material used for isolation together with the corresponding pattern is shown in Table 1.

Table 1. Patterns obtained for the *Armillaria* species identified in the north-west of Spain, host and fungal material.

Species (pattern)	Host fungal	Fungal material
A. mellea (mel 1)	*Camellia* sp., *Vitis* sp.	Rhizomorphs, mycelium
A. mellea (mel 2)	*Actinidia deliciosa, Acacia* sp., *Catanea sativa, Crataegus azarolus, Euonymus japonicum, Hydrangea* sp., *Malus domestica, Magnolia grandiflora, Olea sativa, Prunus domestica, Pinus pinaster, Pinus* sp, *Quercus robur, Quercus suber Salix babilonica, Syringa vulgaris, Salix babilonica, Rhododendron, Vitis* sp.	Rhizomorphs, mycelium, basidiospores
A. gallica (gal 1)	*Acacia melanoxylum, Aesculus hippocastanum, Acer pseudoplatanus, Citrus sinensis, Corylus avellana, Prunus avium, Robinia pseudoacacia, Thuja* sp., *Vitis* sp.	Rhizomorphs, mycelium, basidiospores
A. ostoyae (ost)	*Pinus pinaster, Pinus* sp., *Pinus tabulaephornis*	Rhizomorphs, mycelium, basidiospores
A. cepistipes (cep 1)	*Vitis* sp.	Rhizomorphs

The lack of studies on the identification of *Armillaria* species in Spain led to the assumption that *A. mellea* was the causal agent for all infections. However, the use of this PCR-RFLP technique allowed us to confirm the presence of four *Armillaria* species, even though *A. mellea* is the most frequently encountered. The use of this technique for routine examination will allow a rapid diagnosis of the genus on different hosts and this will help growers to take control measures to reduce or minimise the damage caused by the pathogenic species.

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INVESTIGATIONS ON *HETEROBASIDION* IN CENTRAL AND EASTERN ASIA

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SUMMARY

In order to throw light on the taxonomy of *Heterobasidion* in Eurasia, 48 samples of *H. annosum s. lato* and 32 samples of *H. insulare* from several regions in Asia were investigated with the aid of mating tests and DNA fingerprinting using random amplified microsatellite (RAMS) and M13 minisatellite primers. The results obtained with these methods were in good agreement. Only one species belonging to the *H. annosum* complex was identified from eastern Asia. It is sexually compatible with the European *H. parviporum* (S type), but shows very high compatibility also with the European *H. abietinum* (F type). The strains from the eastern Himalayas (Yunnan) were almost equally compatible with both species; the DNA studies indicated, however, that they are more closely related to the European *H. parviporum*. *H. parviporum* was found to have a wide distribution extending from Europe in the west through southern Siberia to northern China and Japan in the east, and to the eastern Himalayas in the south. *H. annosum s. stricto* (European P type) was only found in the Altai area, southern Siberia, where it causes damage in pine plantations. The bipolar sexual mechanism of *H. insulare* was elucidated. Three intersterility groups of this fungus were identified: one from Taiwan and southern China, one from the eastern Himalayas, and one from northern China. The latter two are very close relatives and only partially intersterile.

Keywords: *Heterobasidion annosum, H. parviporum, H. insulare*, species concept, sexuality, intersterility, distribution

INTRODUCTION

During the last 20 years, the fungus *Heterobasidion annosum* (Fr.) Bref. *s. lato* has been divided into several species and intersterility groups. The following species and groups are clearly distinguishable:

Europe:

- *H. annosum* (Fr.) Bref. *s. stricto* (= European P type) occurs mostly in pine forests. The distribution area includes the whole of Europe, excluding the northernmost forested areas.
- *H. parviporum* Niemelä & Korhonen (= European S type) is distributed throughout central and northern Europe, and attacks *Picea abies* and *Abies sibirica*.
- *H. abietinum* Niemelä & Korhonen (= European F type) occurs on species of *Abies* in southern and central Europe.

North America:

- the N. American S group ('fir group') occurs on coniferous trees belonging to several genera (e.g. *Abies, Picea, Pseudotsuga* and *Sequoiadendron*) in western North America.
- the N. American P group ('pine group') is distributed throughout the North America and attacks mostly pine species.

Australia and adjacent islands:

- *H. araucariae* Buchanan differs in many respects from members of the *H. annosum* complex in the Northern Hemisphere, e.g. it is homothallic and non-pathogenic.

In Asia, the situation is relatively unclear. Within the *H. annosum* complex, only *H. parviporum* has been identified in Northern China (Dai and Korhonen 1998). Another species in eastern Asia is the almost non-pathogenic *H. insulare* (Murray) Ryv. (Fig. 1) Its distribution area extends in the north to the Russian Far East and Japan, in the south to the Philippines, and in the west to India (Buchanan 1988). The sexuality and population structure of this species are not known.

In this study, isolates of *Heterobasidion* from central and eastern Asia were investigated using mating tests and DNA fingerprinting. The Asian isolates were compared with the European and North American representatives of the *H. annosum* complex. The aim of the study was to elucidate the taxonomy and evolution of this genus in Eurasia.

MATERIAL AND METHODS

The material contained representatives of the *H. annosum* complex and *H. insulare*, and originated from the following areas:

	Nr. of samples		
Area	***H. annosum s.l.***	***H. insulare***	**Host**
Altai, southern Siberia	6	-	*Abies sibirica*
	5	-	*Pinus sylvestris*
Kirghizia, central Asia	1	-	*Picea abies*
Heilongjiang province, NE China	-	5	*Abies*
Jilin province, NE China	17	9	*Abies, Picea*
Guizhou province, S. Chine	-	5	*Pinus*
Yunnan province, SW China	13	10	*Abies, Picea, Pinus*
Taiwan	-	2	*Pinus*
Japan	2	1	*Abies*
India	4	-	*Picea, Pinus, Cedrus*
TOTAL	48	32	

The samples primarily consisted of fruit bodies, from which single-spore cultures were isolated. A few samples consisted of a tissue isolate only, from which homokaryotic conidia were isolated. In order to investigate the degree of sexual compatibility between different populations (including the European and North American members of the *H. annosum* complex), a number of homokaryotic isolates representing each population were paired on malt extract agar, and the occurrence of clamp connections in the partners or in subcultures taken from them was checked. In order to investigate the sexual mechanism of *H. insulare*, single-spore isolates were paired with each other and the pairings were investigated daily under the microscope.

Most of the samples were also investigated by DNA fingerprinting using random amplified microsatellite (RAMS) and M13 minisatellite primers. DNA isolation and amplification of the RAMS and M13 fragments were carried out according to Vainio et al. (1998, 200x).

RESULTS

The results obtained with the mating tests and DNA fingerprinting were in good agreement. The following species and intersterility groups were identified:

H. annosum* s. *str. was found only in *Pinus sylvestris* plantations of the Altai area, southern Siberia, where it causes considerable damage. So far this is the easternmost record of this fungus in Eurasia.

H. parviporum was identified from Kirghizia, the Altai area, Jilin province in China and Japan. The *H. annosum s.l.* strains from Yunnan also apparently belong to this species, although they may not be completely compatible with the European strains, and they are also approximately as compatible with the European *H. abietinum* (Fig. 2). However, DNA fingerprinting indicated that the Yunnan strains are distinctly more closely related to the European *H. parviporum* than to *H. abietinum*.

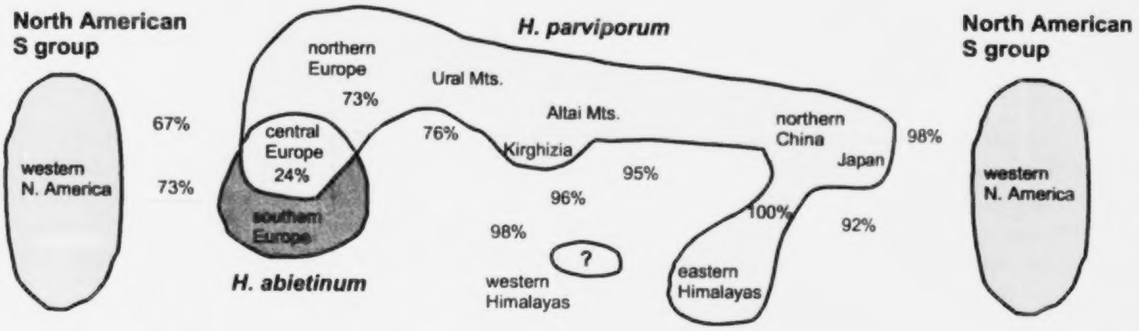
North American S group
western N. America
67%
73%
H. parviporum
northern Europe
73%
central Europe 24%
southern Europe
H. abietinum
Ural Mts.
76%
Altai Mts.
Kirghizia
95%
96%
98%
?
western Himalayas
northern China
Japan
100%
92%
eastern Himalayas
98%
North American S group
western N. America

Figure 1. A schematic picture showing the known distributions of the members of the *H. parviporum* - *H. abietinum* cluster, and the approximate degree of sexual compatibility between some populations. The compatibility is based on the appearance of clamp connections in pairings made in the laboratory, and it does not necessarily indicate that hybrid heterokaryons are competent in nature.

H. insulare proved to be a species complex. Its members have a bipolar mating system. Clamp connections (even double clamps) can be found in homokaryotic mycelia, but compatible matings can be recognised on the basis of their hyphal and mycelial morphology as follows: Initially the hyphae form loose aggregates in the contact zone, from which vigorous clamped hyphae (apparently heterokaryotic) start to grow in different directions. Eventually the demarcation line between the mating partners disappears. The frequency of clamps increases in compatible matings, especially that of double clamps.

Three intersterility groups were identified from the following areas:

'group N' - northern China (Heilongjiang, Jilin) and Japan
'group Y' - south-western China (Yunnan)
'group T' - Taiwan and southern China (Guizhou)

Group T seems to be totally intersterile with groups N and Y, and it was grouped separately in the clustering analysis of the DNA markers. Groups N and Y are very close relatives; they are only partially intersterile, and were grouped together in the clustering analysis. So far little is known about possible differences in the ecology of these groups.

The four *H. annosum s.l.* isolates from India (western Himalayas) showed only a low mating frequency with all the European and Asian species of *Heterobasidion*. However, these isolates had been kept in pure culture for almost 50 years.

DISCUSSION

Five species or intersterility groups belonging to the genus *Heterobasidion* were identified from coniferous forests of central and eastern Asia. Among them, *H. parviporum* has a wide distribution ranging from Europe through southern Siberia to northern China and Japan in the east, and to the eastern Himalayas in the south. In the most extreme parts of the distribution area (Europe and eastern Asia) the representatives of this species show some differentiation from each other. The fungus occurs in forests where species of *Picea* or *Abies* grow. In the Altai area, *H. parviporum* causes damage in managed *Abies sibirica* stands, but it does not seem to be as pathogenic towards *Abies* and *Picea* species of eastern Asia (Dai and Korhonen 1998).

H. annosum s. str. was found only in the Altai area in southern Siberia where it causes damage in *Pinus sylvestris* plantations. It is interesting that this species has so far not been recorded from eastern Asia. Almost all our isolates of *Heterobasidion* from *Pinus* species in this area proved to belong to *H. insulare*.

The sexual system of *H. insulare* was solved, and the fungus was shown to be a species complex. Three intersterility groups were identified from an area that includes China, Japan and Taiwan. All of them have a bipolar mating system, like members of the *H. annosum* complex. In contrast to the *H. annosum* complex, however, *H. insulare* has clamp connections also in homokaryotic mycelia. In this respect it resembles the homothallic species *H. araucariae* (Chase et al. 1985). The phylogenetic position of *H. insulare* seems to be between the *H. annosum* complex and *H. araucariae* (Harrington et al. 1998).

There is some indication that a hitherto unknown intersterility group of the *H. annosum* complex may occur in India, in the western Himalayas. The isolates originating from this area reacted only weakly in the mating tests with other members of the complex. However, the isolates were almost 50 years old (from the years 1954 and 1955) and it is possible that they had partially lost their mating ability. On the other hand, the two *H. parviporum* isolates investigated from Japan were even older (from the years 1942 and 1952) and, in spite of their age, they reacted well in the mating test.

The high diversity of *Heterobasidion* species in south-eastern Asia indicates that much of the variation of the genus originates from this area. For instance, it appears that the diversification of *H. parviporum* and *H. abietinum* may have started in the eastern Himalayas because the local strains of *H. parviporum* also show a very high sexual compatibility with the European *H. abietinum*. However, it is also possible that these species have diverged at a later stage, perhaps in Europe (Garbelotto et al. 1998, Harrington et al. 1998). Some support for both hypotheses can be found from the DNA data available thus far. In case the *H. annosum s.l.* occurring in the western Himalayas does indeed prove to be very different from both *H. parviporum* and *H. abietinum*, the latter hypothesis is then the more probable one.

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POLYMORPHISM WITHIN THE 26S rDNA AND INTERGENIC SPACER (IGS-1) OF WILD AND ARTIFICIAL GENETS OF *ARMILLARIA* SPP. REVEAL PUTATIVE NATURAL HYBRIDS AND PHYLOGENIC RELATIONSHIPS

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PCR products from *Armillaria* genets representing Northern Hemisphere species were produced using 26S (LR12R) and 5S (O-1) ribosomal DNA (rDNA) primers. These external primers and two internal primers were used to produce four overlapping sequenced fragments that provided verification of PCR product ends and conformation of each base position. The high-fidelity sequences provided critical information regarding insertions/deletions (indels) and single nucleotide polymorphisms (SNP) with which to investigate genetic relationships. The partial sequence (ca. 260 bp) of the 26S rDNA was highly conserved. A one-base substitution in this region separated *A. ostoyae*, *A. mellea*, and *A. gemina* from *A. calvescens*, *A. sinapina*, *A. gallica*, *A. nabsnona*, *A. cepistipes*, and NABS X. Three additional substitutions separated *A. mellea* from all other species. The IGS-1 (ca. 600 bp) was highly variable and contained numerous indels and SNPs. The IGS-1 sequence of artificial interspecific hybrids (NABS X and *A. sinapina*, NABS X and *A. cepistipes*, *A. cepistipes* and *A. sinapina*) compared to those of wild genets of these species may reflect gene flow among natural populations. Patterns of specific indel and SNP occurrences among natural populations of *A. ostoyae* suggest that geographical and/or environmental races might be definable. These preliminary results strongly suggest that critical study of high-fidelity sequences of the IGS-1 will contribute significantly to our understanding of phylogeny and population genetics within the genus *Armillaria*.

PHYLOGENETIC RECONSTRUCTION OF NORTH AMERICAN *ARMILLARIA* SPECIES AND RELATED EUROPEAN TAXA BASED ON NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACERS

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New York 13210

The linkage of morphological, genetic, and molecular characters of Armillaria over the past few decades has led to the recognition of intersterile groups designated as "biological species." Data from such studies, especially those using molecular diagnostic tools, have removed a great deal of uncertainty for mycologists and forest pathologists. However, new questions remain to be answered regarding the phylogeny of North American *Armillaria* species and their relationships to their European counterparts, particularly within the "*Armillaria mellea* complex." Our data suggest that North American and European *A. gallica* isolates are not monophyletic. Although North American and European isolates of *A. gallica* may be interfertile, some North American isolates of *A. gallica* are more closely related to the North American taxon *A. calvescens* than to European isolates of *A. gallica*. We find that the increase in genetic divergence has not necessarily paralleled the development of intersterility barriers between isolated populations of *A. gallica*. Though the relationships among some groups within the genus seem clarified, the investigation of geographically diverse isolates has revealed that the relationship between some North American species is still unclear. Further studies are needed to investigate other regions of the genome which may provide a more robust phylogeny of the genus.

Ecology and Biodiversity

ARMILLARIA AND *ANNOSUM* ROOT DISEASES IN A MOUNTAIN PINE (*PINUS MUGO* VAR. *UNCINATA*) STAND IN THE ALPS

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SUMMARY

The cause of tree mortality was investigated in a mountain pine (*Pinus mugo var. uncinata*) forest in the Swiss National Park in the Alps. Recently dead mountain pines in a 2 ha study plot were assessed for root rot and the causal agents were identified. All of the 61 trees examined showed signs of root rot. *Heterobasidion annosum* was detected on 39 trees and *Armillaria* sp. on 28 trees, including 11 trees with both pathogens. The *Armillaria* isolates were identified as *A. borealis* or *A. cepistipes*, both considered as saprotrophs or weak pathogens. All *H. annosum* isolates belong to the P-group, a known primary pathogen of many pine species. The results of this study suggest that root diseases play an important role in the dynamics of these forests with *H. annosum* acting more as a primary pathogen and *Armillaria* sp. as a secondary pathogen.

Keywords: *Pinus mugo var. uncinata*, root disease, *Armillaria cepistipes, Armillaria borealis, Heterobasidion annosum*.

INTRODUCTION

Natural disturbances play an important role in successional processes of forest ecosystems. In the Alps, mountain pine (*Pinus mugo var. uncinata*) behaves as a pioneer tree species, which is often succeeded by the climax tree species larch (*Larix decidua*) and stone pine (*Pinus cembra*). Relatively high tree mortality has been observed in mountain pine forests in the Swiss National Park in the central Alps, which indicates an ongoing successional development in these forests. The patterns of tree mortality and their possible causes have recently been investigated in these forests (Dobbertin et al. 2001). Spatial pattern analysis showed clustering of both living and dead trees suggesting the presence of distinct mortality centers. Root diseases caused by *Armillaria* sp. and *Heterobasidion sp.* were identified as possible causes of the tree mortality observed.

The objective of this study was to identify the species of *Armillaria* and *Heterobasidion* that are involved in the mountain pine mortality observed in this stand.

MATERIAL AND METHODS

The mountain pine stand is located in the Swiss National Park at 1900 m a.s.l. in the central Alps and was not managed since the foundation of the Park in 1914. *Armillaria* sp. and *Heterobasidion* sp. were isolated from the roots of recently dead mountain pine trees as described by Dobbertin et al. 2001. Three main roots near the stem of each tree were assessed for infections by the two pathogens and an attempt was made to isolate a pure culture from each infected root. In addition, the rooting zone of the trees was searched for *Armillaria* rhizomorphs. Pure cultures of *Armillaria* sp. were obtained from the rhizomorphs as described by Rigling et al. 1998. The *Armillaria* isolates were first assigned to somatic incompatibility groups (=genets) by pairing isolates on Shaw and Roth's medium (Harrington et al. 1992). Representative isolates from each genet (at least two isolates, if available) were then identified to species-level by pairing with haploid tester strains (Korhonen 1978) and by using a polymerase chain reaction (PCR)-based identification method (Harrington and Wingfield 1995). To identify species, all PCR products were digested with the restriction enzymes *Alu*I, *Hin*cII (*Hin*dII), and *Mva*1269I (*Bsm*I).

Internal transcribed spacer (ITS)-polymorphism analysis was used to determine intersterility groups of *H. annosum* isolates (Karjalainen and Fabritius 1993). The ITS region was PCR-amplified using the primer pair ITS1/ITS4 and the PCR product was digested with the restriction enzyme *Hin*6I (*Hha*I). The resulting digest was electrophoresed through a 1.5% agarose gel and stained with ethidium bromide. Reference isolates obtained from *Pinus pinaster* (putatively P group) and *Picea abies* (putatively S group) were included in the analysis for comparison. Direct PCR amplification from living mycelium (Harrington and Wingfield 1995) was performed for both *H. annosum* and *Armillaria* sp.

RESULTS

Three main roots of 61 recently dead trees were examined for root rot. A total of 96% of the root samples showed visible symptoms of root rot as indicated by wood decay or discoloration. All of the trees had at least one root with signs of rot. *Armillaria* sp. were identified on 29 trees and *H. annosum* on 39 trees, including 11 trees with both fungi.

A total of 39 isolates of *Armillaria* sp. were isolated from recently dead mountain pine trees and identified to species-level (Table 1). Two *Armillaria* species were found among the isolates. Both identification methods, PCR-RFLP and pairings with haploid tester strains, gave the same results. About one half of the isolates belonged to *A. borealis* and the other half to *A. cepistipes*. Both species showed the same incidence in the rotted roots. In addition, *A. borealis* was preferentially isolated from mycelial fans whereas *A. cepistipes* from rhizomorphs collected in the rooting zone of the sample trees.

Table 1. Number of *Armillaria* isolates from different sources obtained from recently dead mountain pine trees.

Source of isolates	No. of isolates[1]	*A. borealis*	*A. cepistipes*
Mycelial fans[2]	12	10	2
Root rot (decaying wood)	16	8	8
Rhizomorphs	11	2	9
Total	39	20	19

[1] Isolates were obtained from a total of 28 trees with 19 trees yielding one isolate, seven trees yielding two isolates, and two trees yielding three isolates (i.e., one isolate from each source).

[2] Mycelial fans were collected from the stem basis and the roots of the sample trees.

The *A. borealis* isolates were assigned to two genets and the *A. cepistipes* isolates to three genets (Table 2). One large *A. borealis* genet was identified, which comprised most of the isolates of this species. This genet was found on 11 sample trees which all showed signs of infections as indicated by the presence of mycelial fans and/or by the successful isolation of *Armillaria* from the rotted roots. The majority of the *A. cepistipes* isolates also belonged to one large genet (B1), which was isolated from 11 recently dead trees. Seven of these trees were infected by this genet while four trees had only rhizomorphs in their rooting zones, without signs of an *Armillaria* infection. Genet B2 was isolated from three trees, one infected and two showing only rhizomorphs. Although this genet was only represented by four isolates from three trees, the maximum distance among the isolates was relatively large. These isolates may represent ramets of a large genet without spatial contiguity of the isolates. The genet B3 was represented by only one isolate from a single tree. Multiple isolates from different sources or roots were obtained from nine trees. In all these cases, all the isolates belong to the same genet.

All six trees where *Armillaria* sp. were only present as rhizomorphs in the soil showed root infections by *H. annosum*.

Table 2. *Armillaria* genets identified in the study plot.

***Armillaria* genet**	**No. of trees**	**No. of trees infected**	**No. of trees with only rhizomorphs**	**No. of isolates**	**Max distance (m) among isolates**
A. borealis A1	11	11	0	16	98
A. borealis A2	2	2	0	4	15
A. cepistipes B1	11	7	4	13	125
A. cepistipes B2	3	1	2	4	117
A. cepistipes B3	1	1	0	1	n.a.
Total	28	21	6	39	

n.a. = not applicable

H. annosum was identified in the roots of 39 recently dead mountain pine trees. Most of the trees had more than one root infected by the fungus (Table 2). PCR-based identification was performed with 14 isolates, all from different trees. All the isolates showed the same restriction pattern as the reference isolate of the P group. Fig. 1. shows the results of 11 isolates.

Table 3. Incidence of *H. annosum* in the main roots of recently dead mountain pine trees.

No. of roots with *H. annosum*	**No. of trees**
0	22
1	11
2	13
3	15
Total trees with *H. annosum*	39

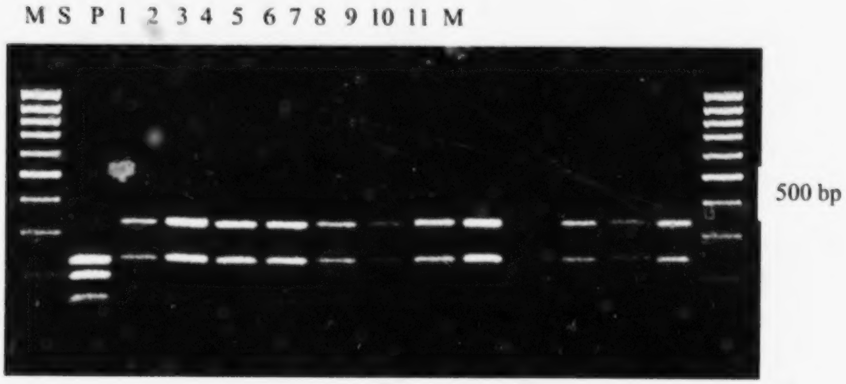

Figure 1. Agarose gel electrophoresis of *H. annosum* PCR products amplified by primers ITS1/ITS4 and digested with the restriction enzyme *Hin*6I (*Hha*I). M, 100 bp DNA ladder; S, reference isolate of the S group; P, reference isolate of the P group; 1-11, *H. annosum* isolates from mountain pine.

DISCUSSION AND CONCLUSION

Dobbertin et al. 2001 provided evidence that the high tree mortality observed in a mountain pine stand in the Swiss National Park was impacted by *Armillaria* sp. and *H. annosum*. The spatial and quantitative data of that study suggested that *H. annosum* was a more important pathogen than *Armillaria* sp. The results of the present study further support this conclusion. The two *Armillaria* species identified, *A. borealis* and *A. cepistipes*, are generally considered as saprotrophs or weak secondary pathogen (Guillaumin et al. 1993). *A. ostoyae*, the most pathogenic *Armillaria* species of conifers (Guillaumin et al. 1993), was not detected in this study. *A. ostoyae* has been reported on mountain pine in the Pyrénées, where it behaves as a primary pathogen (Durrieu et al. 1985).

Two causal agents, *A. cepistipes* and *A. borealis*, of Armillaria root disease of mountain pine were identified. Both *Armillaria* species were isolated from mycelial fans and roots of recently dead mountain pines. These findings indicate that both species act to a certain degree as pathogens in this forest. Nevertheless, *A. borealis* appears to behave more pathogenic than *A. cepistipes*, as indicated by the fact that it was more frequently isolated from mycelial fans than *A. cepistipes*. The latter species, on the other hand, was more frequently isolated from rhizomorphs collected in the rooting zone of the trees, most of which were infected by *H. annosum*. With these rhizomorphs, *A. cepistipes* eventually is able to saprophytically colonize the root system of the trees after it has been killed by *H. annosum*.

A. cepistipes is the dominating soil-rhizomorph producing *Armillaria* species in Switzerland, which frequently occurs in coniferous forests (Rigling et al. 1998). It was found to be common between 600 - 1600 m. a.s.l. and apparently is rare above this altitude. The mountain pine stand at 1900 m a.s.l. represents one of the highest habitat reported for *A. cepistipes* in Europe. Compared to *A. cepistipes*, *A. borealis* is rare in Switzerland (Rigling et al. 1998). This species has been found at low and moderate altitude in central Europe (Guillaumin et al. 1993). The present study demonstrates that *A. borealis* also occurs at high altitude in the Alps. Two genets of this species were identified. The larger genet most likely expands beyond the border of the study plot and its area and age remains to be determined.

In contrast to the *Armillaria* species identified, the P group of *H. annosum* is known as primary pathogen, which can attack and kill different species of pine, including mountain pine (Korhonen et al. 1998). Almost two thirds of the recently dead mountain pine trees were infected by *H. annosum*. Trees infected by *H. annosum* were on average larger and had a larger growing space than trees infected by *Armillaria* (Dobbertin et al. 2001). Mortality of mountain pine in this forest also occurs in the natural regeneration. Among ten recently dead trees examined, nine were infected by *H. annosum* and only one by *Armillaria* sp. (data not shown). These results indicate that *H. annosum* is acting more as a primary pathogen whereas the two *Armillaria* species as secondary pathogens.

Root diseases apparently influence the dynamics of this mountain pine forests. The precise role of the root diseases in the successional process, however, needs to be determined. By causing significant mortality in mountain pine, the diseases could favor the establishment of other tree species, like larch and stone pine. On the other hand, by producing expanding mortality centers (Dobbertin et al. 2001) they could again favor the regeneration of the pioneer species mountain pine in the developing openings.

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THE INFLUENCE OF PLANT SERIES AND PLANT ASSOCIATION GROUPS ON THE INCIDENCE AND SEVERITY OF ROOT DISEASES IN SOUTHWEST OREGON FORESTS

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SUMMARY

Southwest Oregon forests are highly diverse. Forest types encountered include low elevation, dry, oak-pine woodlands, mixed conifer-evergreen hardwoods, Douglas-fir dominated coastal stands, and high elevation true fir forests. 327 permanent monitoring plots, originally established to develop climax vegetation classes for Southwest Oregon, were examined for presence and severity of a wide range of forest insects and pathogens including *Phellinus weirii*, *Armillaria ostoyae*, and *Heterobasidion annosum*. Root diseases were not detected on plots in the Oregon White Oak, Ponderosa Pine, Jeffrey Pine, Lodgepole Pine, or Western White Pine Climax Series. Root diseases were present in the Tanoak (9% of plots examined), Douglas-fir (5%), Port-Orford-Cedar (42%), Mountain Hemlock (43%) White Fir (47%) Shasta Red Fir (40%) Western Hemlock (40%) and Pacific Silver Fir (67%) Series. Within Plant Series, incidence of root disease differs among Plant Association Groups. For example, root diseases were detected in only one of eight Plant Association Groups within the Douglas-fir Series and five of six Plant Association Groups in the Western hemlock Series. Root disease severity was rated on each plot using a 0-9 scale. Average severity ratings on plots with root disease (rating ≥ 2) ranged from 2.2 in the Shasta Red Fir Series to 3.6 in the Pacific Silver Fir Series. Severity differed among Plant Association Groups within Series. Root disease severity ratings were higher for plots with Armillaria root disease, laminated root rot, and Port-Orford-cedar root disease than for plots with Annosus root disease or black stain root disease. Results of this survey indicate that root pathogens are important, are widely distributed, and have substantial impacts in mature forest stands in Southwest Oregon. Occurrence and impacts differ among Plant Series and Plant Association Groups. Based on this evaluation, Plant Association Groups in the Port-Orford-cedar, White Fir, Shasta Red Fir, Pacific Silver Fir, Western Hemlock, and Mountain Hemlock Series have the greatest overall amounts of root disease in mature stands, probably because of more conducive site and environmental conditions as well as greater components of susceptible hosts in the stands. Series where root diseases were not present in mature stands occur at lower elevations on dry sites, on ultramafic soils, and/or have high components of hardwood species, pines, and incense-cedar which are relatively resistant to the root disease fungi encountered.

Keywords: *Phellinus weirii*, *Armillaria ostoyae*, *Heterobasidion annosum*, Plant Series, Plant Association Groups

INTRODUCTION

Southwest Oregon is very different from other forested areas in the Pacific Northwest and in fact is one of the ecologically most diverse regions in North America (Atzet and Martin 1991). Unique features that contribute to this diversity include an extremely varied geologic history, an associated abundance of very different soil types, a climate that is Mediterranean rather than temperate, a substantial role of fire in forest succession, and occurrence of important junctures between several north-south and east-west running mountain ranges that has led to significant migrations of plant and animal species into the region from elsewhere. Soutnwest Oregon forests contain 24 species of conifers and 14 hardwood tree species as well as an especially rich array of shrubs and herbaceous plants, a substantial number of which reach the northern, southern, or western extent of their ranges in Southwest Oregon or are endemic to the region.

Forest ecologists in Southwest Oregon classify plant communities based on potential natural vegetation (Atzet et al. 1996). The potential natural vegetation for a site is the vegetation that would be present under climax conditions, conditions that would develop without natural or human-caused disturbances. The climate of Southwest Oregon favors a frequent fire disturbance regime and this coupled with other disturbances, especially forest management activities by humans, results in the actual occurrences of climax vegetation being quite rare. Most forest stands have been burned several times or have had some harvesting, are multi-aged, and are in early or mid successional stages. The oldest trees are commonly less than 300-years-old. For such stands, potential natural vegetation is inferred using information on existing younger successional vegetation and knowledge of successional pathways.

The potential vegetation classification system has two levels of which the broader divisions are Plant Series and the finer divisions are Plant Associations. Series are described by the dominant, most shade-tolerant, regenerating species on the site. Plant Associations are described primarily by the presence, absence, and relative abundance of plant species. Environmental variables, including soil, are also used in the classifications and often reflect the pattern of vegetation. Species presence and abundance result from environmental gradients. Classification attempts to find plant responses to natural gradients such as slope, slope position, aspect, soil type, temperature, and moisture. To facilitate meaningful classification at the landscape scale, similar Plant Associations have been combined into Plant Association Groups.

Plant Series present in Southwest Oregon are the Sitka Spruce (*Picea sitchensis*), Oregon White Oak (*Quercus garryana*), Ponderosa Pine (*Pinus ponderosa*), Tanoak (*Lithocarpus densiflorus*), Douglas-fir (*Pseudotsuga menziesii),* Western Hemlock (*Tsuga heterophylla*), Western Redcedar (*Thuja plicata*), Port-Orford cedar (*Chaemacyparis lawsoniana*), Jeffrey Pine (*Pinus jeffreyii*), White Fir (*Abies concolor*), Lodgepole Pine (*Pinus contorta*), Shasta Red Fir (*Abies magnificae* var. *shastensis*), Pacific Silver Fir (*Abies amabalis*), Western White Pine (*Pinus monticola*), and Mountain Hemlock (*Tsuga mertensiana*) Series. The Sitka Spruce and Western Redcedar Series have very limited distributions and were not included in the present evaluation. Each Series contains one to several Plant Association Groups. The 13 Series investigated in this evaluation contain among them 42 Plant Association Groups.

When forest ecologists were first developing the potential vegetation system for Southwest Oregon in the late 1970s and early 1980s, they established 1200 plots in relatively undisturbed, mature stands that they felt best represented the various Plant Series and Plant Associations. These plots were not located randomly. Rather, location was dictated by occurrence of stands with the right histories and characteristics for the analysis. Nevertheless, the plots are quite well distributed across the three Southwest Oregon National Forests. Data from the Southwest Oregon ecology plots have been widely used in forest planning and management in the region. Not only has the classification system proven important for ensuring that vegetation management prescriptions are appropriate to individual sites and landscapes, but data on fire histories, numbers of snags, and amount of down wood from the ecology plots has been extensively used to develop management policies.

Unfortunately, no data on forest insects or diseases were originally collected in the Southwest Oregon ecology plots. The purpose of the present evaluation was to revisit a representative sample of the plots and collect this kind of information. Our objectives were to use plot data to get an idea of distribution of significant forest insects and pathogens in mature stands in Southwest Oregon, evaluate the magnitude of their impacts in such stands, and investigate the relationships between Plant Series and Plant Association Groups and the important forest insects and diseases. This evaluation, along with data from other surveys and large-scale forest inventories, will help us provide meaningful insect and disease information that is tied directly to the vegetation classification system used as the basis for silvicultural prescriptions. This paper reports on our results for the five major root pathogens found in the area: *Armillaria ostoyae*, cause of Armillaria root disease, *Heterobasidion annosum*, cause of Annosus root disease, *Phellinus weirii*, cause of laminated root rot, *Phytophthora lateralis*, cause of Port-Orford-cedar root disease, and *Leptographium wageneri*, cause of black stain root disease.

METHODS

We randomly selected 327 of the 1200 ecology plots for insect and disease evaluation. Plots chosen represented a 20 to 25% sample of all plots in each Plant Series and Plant Association Group. Plots were located in the field using the original ecologists' location maps and descriptions.

In each case after the plot center tree was located, we established a 20-basal area factor variable radius plot that was centered 10 feet to the north. All trees greater than 12.7 cm diameter at breast height in this plot were recorded by species, diameter, and condition (live, dead, standing, broken, down). All were carefully examined for signs and symptoms of infection by root pathogens as well as evidence of any other detectable insects or pathogens. At the same center point, we also established a 0.004 ha circular fixed area plot within which we recorded the same information for all trees less than 12.7 cm dbh but greater than 15 cm tall. We also established two 15.3 meter-long down wood transects starting at the plot center and running due north and due east. We recorded diameter and condition of all down trees and pieces of down wood 7.6 cm in diameter or greater that were intersected by each transect. Wherever possible, we recorded pathogens or insects that apparently contributed to the wood being on the ground. Finally, we gave a root disease severity rating to a 0.02 ha circular area surrounding plot center (Table 1) using the technique developed by Hagle (1985).

Table 1. Root disease severity ratings.

Rating	Description
0	No evidence of root disease visible within 15.3 meters of the plot.
1	Root disease present within 15.3 meters of the plot but no evidence of root disease on plot.
2	Minor evidence of root disease on plot, such as a suppressed understory tree killed by root disease or a minor part of the overstory showing signs of infection. Little or no detectable reduction in canopy closure or volume.
3	Canopy reduction evident, up to 20%, usually as a result of the death of 1 codominant tree on an otherwise fully stocked site. In the absence of mortality, numerous trees showing symptoms of root disease.
4	Canopy reduction 20% to 30% as a result of root disease. Snags and downed trees removed from canopy by disease as well as live trees showing symptoms of disease contribute to impact.
5	Canopy reduction 30-50% as a result of root disease. At least half of the ground area of plot considered infested. Plots representing mature stands with half their volume in root disease-tolerant species usually do not go much above a severity rating of 5 due to the ameliorating effect of the root disease-tolerant species.
6	50-75% canopy reduction as a result of root disease with most of the ground area considered infested.
7	At least 75% canopy reduction. Plots which reach this severity level usually are occupied by only the most susceptible species. There are very few of the original overstory trees remaining although infested ground is often densely stocked with regeneration of susceptible species.
8	The entire plot falls within a definite root disease pocket with only 1 or very few overstory trees of susceptible species present.
9	The entire plot falls within a definite root disease pocket with no overstory trees of the susceptible species present.

RESULTS

Twenty-nine percent of all ecology plots sampled had trees affected by root pathogens. Root disease occurrence differed among Plant Association Groups and Plant Series. Root diseases were not detected on plots in the Oregon White Oak, Ponderosa Pine, Jeffrey Pine, Lodgepole Pine, or Western White Pine Series. Root diseases were present in the Tanoak (9% of plots examined), Douglas-fir (5%), Port-Orford-Cedar (42%), Mountain Hemlock (43%) White Fir (47%) Shasta Red Fir (40%) Western Hemlock (40%) and Pacific Silver Fir Series (67%). Relatively few plots with root diseases were found in any of the Plant Association Groups in the

Douglas-fir and Tanoak Plant Series (Table 2). Relatively large numbers of plots with root disease were found in many of the Plant Association Groups in the Port-Orford-Cedar, White Fir, Shasta Red Fir, Pacific Silver Fir, Western Hemlock, and Mountain Hemlock Plant Series (Table 2). On plots where root diseases occurred, root disease severity ratings ranged from 2 to 9. Overall average root disease severity ratings were lowest for plots in the Tanoak, Shasta Red Fir and Mountain Hemlock Series, intermediate in the Western Hemlock, Douglas-fir and White Fir series, and highest in the Pacific Silver Fir and Port-Orford-Cedar Series (Table 3). Root disease severity ratings were higher for plots with Armillaria root disease, laminated root rot, and Port-Orford-cedar root disease than for plots with Annosus root disease or black stain root disease.

Table 2. Percentage of plots in each Plant Series with root disease by pathogen.

Series*	*A. ostoyae*	*P. weirii*	*H. annosum*	*P. lateralis*	*L. wageneri*
Lodgepole Pine (7)	0	0	0	0	0
Ponderosa Pine (2)	0	0	0	0	0
Oregon White Oak (4)	0	0	0	0	0
Port-Orford-Cedar (13)	17	0	0	25	0
Douglas-fir (41)	0	2	0	0	2
Tanoak (45)	4	0	0	2	0
Western White Pine (3)	0	0	0	0	0
Western Hemlock (42)	17	10	17	0	0
White Fir (107)	31	5	12	0	0
Shasta Red Fir (15)	27	0	20	0	0
Pacific Silver Fir (15)	41	7	0	0	0
Mountain Hemlock (21)	38	0	10	0	0

*Number in parentheses is the number of plots sampled.

Table 3. Mean root disease severity rating by Plant Series for plots with ratings ≥ 2.

Series	Rating*
Port-Orford-Cedar	3.5 (2 to 6)
Douglas-fir	3.0 (2 to 4)
Tanoak	2.7 (2 to 3)
Western Hemlock	3.2 (2 to 7)
White Fir	3.3 (2 to 9)
Shasta Red Fir	2.2 (2 to 3)
Pacific Silver Fir	3.6 (2 to 5)
Mountain Hemlock	2.7 (2 to 4)

* Mean with range in parentheses

Among Plant Association Groups that contained hosts susceptible to *A. ostoyae*, 52% had trees affected by the pathogen. Less than 1 to 33% of susceptible host trees in these Plant Association Groups were infected (average = 5%). Percentage of trees infected by *A. ostoyae* was higher in the wetter Plant Association Groups than in the drier ones, and Plant Association Groups in the Pacific Silver Fir and White Fir Series had higher percentages of trees infected than other Series.

Among Plant Association Groups that contained hosts susceptible to *H. annosum*, 43% had trees affected by the pathogen. One to 16% of susceptible host trees in these Plant Association Groups were infected (average = 4.6%). Percentage of trees affected by *H. annosum* was greater in Plant Association Groups that had stumps or numerous broken trees than in Plant Association Groups that lacked such infection foci. Some Plant Association Groups in the Pacific Silver Fir Series had the highest percentages of trees infected. Some Plant Association Groups in the White Fir and Mountain Hemlock Series also exhibited fairly high numbers of trees infected by *H. annosum* but less than with the Pacific Silver Fir Series.

Among Plant Association Groups that contained hosts susceptible to *P. weirii*, 14% had trees affected by the pathogen. One to 39% of susceptible host trees in these Plant Association Groups were infected (average = 11%). Percentage of trees infected by *P. weirii* tended to be higher in the dry to mesic Plant Association Groups than in the wet ones, and Plant Association Groups in the White Fir Series and one in the Douglas-fir Series had the highest percentages of trees infected by *P. weirii*.

Among Plant Association Groups that contained hosts susceptible to *P. lateralis*, 31% had trees affected by the pathogen. Five to 78% of susceptible host trees in these Plant Association Groups were infected (average = 50%). Percentage of trees infected by *P. lateralis* was high in Plant Association Groups where plots with hosts were located in wet areas, and, in addition to Plant Association Groups in the Port-Orford-cedar Series, one in the Tanoak Series had a high percentage of hosts infected.

Only one Plant Association Group that contained hosts susceptible to *L. wageneri* had trees affected by the pathogen. In that Plant Association Group, one in the Douglas-fir Series, 2% of host trees were infected.

DISCUSSION

Results of this survey indicate that root pathogens are important, are widely distributed, and have substantial impacts in mature forest stands in Southwest Oregon. Occurrence and impacts differ among Plant Series and Plant Association Groups. Based on this evaluation, Plant Association Groups in the Port-Orford-Cedar, White Fir, Shasta Red Fir, Pacific Silver Fir, Western Hemlock, and Mountain Hemlock Series have the greatest overall amounts of root disease in mature stands, probably because of more conducive site and environmental conditions as well as greater components of susceptible hosts in the stands. Series where root diseases were not present in mature stands occur at lower elevations on dry sites, on ultramafic soils, and/or have high components of hardwood species, pines, and incense-cedar which are relatively resistant to the root disease fungi encountered.

The relatively high overall incidence and large amounts of root diseases found in our evaluation are especially interesting in view of the way that the ecology plots were originally selected. Plots were specifically chosen to be free of disturbance or at least "relatively undisturbed." Areas with evidence of harvesting or recent fires were avoided as much as possible and forested areas with essentially full canopy cover were preferentially selected. Areas with openings were discriminated against. Therefore, areas where root pathogens would be expected to have their greatest impacts were probably never selected for ecology plot locations. This suggests that, as an overall estimate of root disease in mature Southwest Oregon stands, results from our survey are conservative, especially for those diseases that cause large amounts of mortality.

A. ostoyae is common throughout western North America on many hosts (Hadfield et al.1986; Hansen and Lewis 1997). *A. ostoyae* has been reported to be especially successful on stressed trees (Shaw and Kile 1991) so its frequency in dense, old stands like many of those encountered in the evaluation was not unexpected. However, it also is known that *A. ostoyae* can be extremely virulent in some cases, especially on true firs where it frequently affects trees in all vigor classes (Hadfield et al. 1986; Shaw and Kile 1991). Where we are interested in interpolating our results to all mature Southwest Oregon stands, we likely underestimated the latter kind of impact for *A. ostoyae* in the ecology plots.

P. weirii is common in much of western North America north of the California border (Hadfield et al. 1986; Hansen and Lewis 1997; Thies and Sturrock 1995). It can infect many hosts but is most prominent on Douglas-fir, true firs, and mountain hemlock (Hadfield 1985). Evidence strongly suggests that *P. weirii* affects susceptible host species irrespective of vigor (Hansen and Goheen 2000). In fact, it is frequently encountered in host trees growing on the most productive sites. *P. weirii* is an especially aggressive pathogen that, where established, usually causes many hosts to die, often in relatively large infection centers (Hadfield 1985). For all mature stands, we certainly underestimated occurrence and affects of *P. weirii* in this evaluation because openings of the type it often creates were not sampled. This was especially true in stands containing some hosts such as mountain hemlock.

At least two strains of *H. annosum* are common in much of western North America on a number of hosts (Hadfield et al. 1986; Hansen and Lewis 1997). In Southwest Oregon only the S-type of *H. annosum* that affects true firs and hemlocks is encountered. *H. annosum* spreads long distances via windborne spores that infect freshly cut host stump surfaces and newly created wounds on hosts. Infection centers develop around infected stumps or wounded trees as the pathogen grows across root contacts into surrounding host trees. Because of its relationship with stumps and wounds, build-up of *H. annosum* is often strongly associated with forest management activities or other disturbances that injure trees (Otrosina and Scharpf 1989). The quite high level of *H. annosum* that we encountered in the Southwest Oregon ecology plots was a surprise to us given the relatively undisturbed character of the plots. However, it can probably be explained. There were one or a few stumps in or near a number of the plots. Even in forest stands that are very hard to access, it is difficult to find any substantial areas in Southwest Oregon where at least a few trees were not harvested in salvage operations in the past. Also, some amount of wounding caused by falling trees occurred in many plots. It appears evident that in Southwest Oregon, *H. annosum* centers do not require large numbers of stumps or wounded trees to become established. Even a single stump or wound must often be sufficient.

P. lateralis is an exotic pathogen that was introduced into the rather small natural range of Port-Orford-Cedar in the 1950s (Hadfield et al. 1986; Hansen and Lewis 1997). It requires very moist conditions around host roots for infection to occur and is mostly active where hosts are growing in or near streams, drainages, or flooded areas. When hosts occur in such suitable sites, *P. lateralis* is extremely virulent and kills trees very rapidly (Hansen et al. 2000). In our evaluation, *P. lateralis* was common and had high impacts on most ecology plots that contained hosts and were on suitably moist sites. Even though original plot selection may have discriminated against locations with Port-Orford-Cedar root disease, the rapid spread of *P. lateralis* in water, the very low amount of resistance to the exotic pathogen in host populations, and the rapidity with which infected hosts die probably allowed us to get a reasonable reading on the distribution and impacts of *P. lateralis* relative to distribution and impacts for all mature Southwest Oregon stands. The pathogen undoubtedly became established and killed many host trees on sampled sites where Port-Orford-Cedars appeared to be healthy when the ecology plots were originally established 20 to 25 years ago.

L. wageneri occurs at a number of locations throughout western North America (Hadfield et al. 1986; Hansen and Lewis 1997). The fungus has three strains with rather exacting host requirements (Harrington and Cobb 1988). The strain that infects Douglas-fir, *L. wageneri* var *pseudotsuga*, is most common in young Douglas-fir plantations in northern California and Southwest Oregon. It usually becomes established in areas where hosts are under stress, develops rapidly in the early years of stand development, and has very little impact in stands that are 30 years old or older (Hessburg et al. 1995). We were quite surprised to find *L. wageneri* in any of the mature stands investigated in the ecology plots, but believe that the very low occurrence and impacts found in the evaluation would be typical for mature stands throughout Southwest Oregon.

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BIODIVERSITY IN MONOCULTURES: THE SITKA SPRUCE STUMP

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SUMMARY

Concepts of biodiversity tend to focus on the macro-environment and highly visible plants, animals, and habitats. This view, however, is incomplete as it does not include the high numbers of micro-organisms present in all ecosystems. Where fungi currently are included in estimates of diversity, data frequently depend on the identification of fruiting bodies, which ignores the microfungi component. Traditional plantation management practices have been criticised by environmental groups for promoting monocultures which appear to be species deficient and discourage biodiversity in the generally accepted sense. This paper describes the total microbial biodiversity found in forest monocultures centred on Sitka spruce plantations in the UK. Stumps of certain ages may contain high numbers of fungal species, particularly mitosporic fungi and hymenomycetes. Ascomycota, Oomycota and Zygomycota are present in relatively small numbers. Bacteria are abundant, although current estimates are undoubtedly low, relying entirely on the enumeration of culturable organisms. Decay-causing hymenomycetes usually dominate in stumps of 12 months in age and older, but are frequently found in association with bacteria which may have positive and negative influences on the progress of decay.

Diversity of decay fungi and stump/wood colonising bacteria in more natural forests growing in similar environmental zones is unlikely to be significantly different. It is proposed that different groups of micro-organisms occupying spruce stumps contribute greatly to the biodiversity found in managed plantations and should be included in such assessments.

Keywords: Sitka spruce, thinning stumps, microbial diversity, fungi, bacteria

INTRODUCTION

Recent international agreements made in Helsinki, Rio and Kyoto, obligate signatory countries to manage forests in a sustainable manner to optimise biodiversity. Forest certification and local action plans have added to the pressure on forest managers to increase the diversity of forests under their jurisdiction. In order to demonstrate any changes in diversity resulting from the adoption of more benign management practices it is necessary to gather baseline data on the levels of diversity present in forests under different forms of management. Diversity, however, is extremely difficult to estimate with any degree of accuracy, and in many situations, 'indicators of diversity', such as the amounts of deadwood present, vertical stand structure, or the co-occurrence of particular species (e.g. Landres et al. 1988; Ferris and Humphrey 1999) are used. Such methods have relied heavily on estimates of numbers of larger organisms: invertebrates, birds, bryophytes and vascular plants. Where micro-organisms are included, for example the fungi, quantitative estimates are often based on records of fruit bodies, most of which are ephemeral (e.g. Sippola and Renvall 1999). Fungi with indistinct or microscopic thalli and fruiting structures, are not usually considered. Moreover, prokaryotes have rarely been included (cf. Torsvik et al. 1990; Amann and Luwig 2000), possibly because of the scarcity of relevant expertise and the effort required to gain a true picture of the number and species of bacteria present in a given niche (Roselló-Mora and Amann 2001). As prokaryotes are numerically, and probably in terms of species also, the most abundant organisms present in any given niche, their exclusion from the process threatens the validity of many claims made for estimates of diversity in different ecosystems.

After the First World War, UK forest policy aimed to develop a strategic timber reserve in as short a time as possible, and in the early-mid 20th Century, large areas of the uplands were planted with exotic species, principally Sitka spruce (*Picea sitchensis*) and lodgepole pine (*Pinus contorta*). The value of these introduced species for biodiversity is controversial. Some reports have suggested that plantations reduce the diversity of areas, compared with the habitats the forests replace (Ratcliffe and Thompson 1989), although other studies have given more positive outlooks on the benefits of plantation forestry for wildlife (Petty et al. 1995; Humphrey et al. in press). It has become clear, however, that diversity based on estimates of larger organisms can be high in plantations of exotic species, particularly in mature/over-mature stand types (Humphrey et al. in press).

The amount of standing and fallen deadwood present in a forest area is indicative of the level of human disturbance (Hansson 1992) and is used as an indicator of diversity, as these niches can support wide varieties of organisms (see Sippola and Renvall 1999). Deadwood may sequester a large proportion of the carbon fixed from the atmosphere (Krankina and Harmon 1995). Stumps left in thinning and clear-felling operations are abundant in managed forests and have some potential to mirror the role of deadwood in unmanaged forests. Stumps have received little attention in biodiversity studies, although much work on *Heterobasidion* infections has focused on this niche as the site of primary infection (Redfern and Stenlid 1998). This paper describes the diversity of fungi and culturable bacteria occurring in stumps of Sitka spruce of increasing age in upland plantations in Scotland. The significance of stump micro-organisms in the biodiversity of the Sitka spruce plantation is discussed.

MATERIALS AND METHODS

Sites: Fungi and bacteria were isolated from a range of Sitka spruce (*Picea sitchensis*) plantations in North-East Scotland. All plantations were growing on mineral soils. For fungal work, stumps were produced by felling to waste; surfaces were not treated with urea. Sampling was carried out at 0, 7 and 28 days, 12, 16, 48 and 53 months after felling. Four 30 mm thick serial discs were removed from each stump, placed in clean plastic bags and returned to the laboratory for isolation work. Bacteria were isolated from different points in single 100 mm thick discs taken from stumps cut 1-10 years before sampling.

Isolation and Identification of Fungi: Stump discs were processed as described by Morrison & Redfern (1994), wrapped individually in newspaper and incubated in clean plastic dustbins outdoors at ambient temperatures. During the first 4 days of incubation, discs were removed temporarily to extract cores. After 7-10 days incubation, the discs were examined under a dissecting microscope and any fruiting structures, mycelium or staining noted.

Fungal culture: Cores were extracted from discs using a 5 mm diam. increment borer, taking three equidistant cores from both the heartwood and sapwood. The outer layers of the cores were removed, and two 5 mm^2 chips from the centre of each core transferred to Petri dishes containing one of the following media: 2% malt extract agar, 2% malt extract antibiotic agar (containing 50 mg.l^{-1} penicillin-G, 100 mg.l^{-1} streptomycin sulphate and 50 mg.l^{-1} chloramphenicol) , or Kuhlman and Hendrix selective medium (Kuhlman and Hendrix, 1962). Petri dishes were sealed with Nescofilm and incubated in the dark at 20±2°C for 1-12 weeks, with observations made at 7-day intervals. Any fungal growth emerging from wood chips was sub-cultured to fresh 2% malt extract agar.

Identification of fungi: Characteristic fungal structures visible under the dissecting microscope, including conidiophores and cords, were noted on disc surfaces. Cultured fungi were identified from microscopic characteristics (Ainsworth et al. 1973 a,b; Ellis 1971; 1976; Ellis and Ellis 1985; Stalpers 1978).

Isolation of Bacteria: Methods used in the isolation and characterisation of bacteria are detailed elsewhere (Murray and Woodward this volume). For enumeration, wood tissues (3 cm^3) were removed aseptically from various positions in the stump discs, placed in 10 ml sterile saline solution (0.9% NaCl) and dilution plated (0.1 ml aliquots) onto Reasoner and Geldreich (1985) R2A medium, supplemented with 50 mg.l^{-1} cycloheximide. Cultures were incubated at 20±2°C and numbers of colony-forming units (CFU) estimated at 24 h intervals over 7 days.

Estimation of bacterial diversity: Types of bacteria were crudely estimated on Petri dish cultures showing suitable separation of CFUs on the basis of colony shape, surface characteristics, size, consistency, colour and opacity. Diversity was estimated using the Shannon-Weaver index (Shannon and Weaver 1949) and, for comparison, Simpson's diversity index (Simpson 1949)

Statistical analysis: Linear regression analysis and x^2 test were used to determine the significance of correlations between numbers of micro-organisms isolated and time following felling, or depth within the stump.

RESULTS

Fungal diversity: Total numbers of fungi found in different phyla are shown in Figure 1. Mitosporic species were the most abundant fungi overall. Few Ascomycota were identified, although assuming that most species classified as yeasts and mitosporic species were Ascomycota, then this phylum clearly was the most abundant over the 4.5 years of stump age studied. Numbers of Basidiomycota species increased significantly with time ($P>0.05$); species in other phyla showed no significant changes with increasing stump age.

Most fungi isolated were identified at least to genus level (Table 1). Many Ascomycota and mitosporic fungi were found at only one sampling time. *Heterobasidion annosum* was the first hymenomycete found, initially isolated from stumps 7 days after cutting. *Bjerkandera adusta* and *Melanotus proteus* were established in stumps within 28 days, and other hymenomycetes colonised between this time and 16 months after cutting.

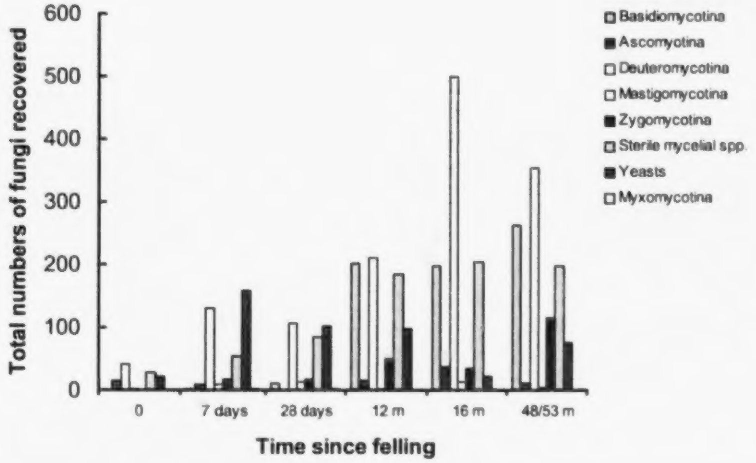

Figure 1. Total numbers of fungi in different phyla isolated from Sitka spruce stumps over 53 months from felling. Numbers of yeast-like organisms and sterile mycelia are presented as separate groups.

Table 1. Times of isolation of identified fungi from stumps of Sitka spruce.

Isolate identification	Period of isolation					
	0	**7 days**	**28 days**	**12 months**	**16 months**	**48/53 months**
Basidiomycota						
Bjerkandera adusta			—	—	—	—
Gleophyllum spp.				—	—	—
Heterobasidion annosum		—	—	—	—	—
Melanotus proteus			—	—	—	—

Isolate identification	Period of isolation 0	7 days	28 days	12 months	16 months	48/53 months
Peniophora pithya				—	—	—
Polyporus abietinus					—	—
Resinicium bicolor					—	—
Sistotrema brinkmannii				—	—	—
Stereum sp.					—	—
Ascomycota						
Ascocoryne sp.						—
Nectria fuckeliana		—	—	—	—	
Nectria inventa		—				
Mariannaea elegans		—				
Ophiostoma piceae				—	—	—
Pseudoeurotium zonatum	—					
Mitosporic fungi						
Acremonium butyri	—				—	
Arthroderma sp.	—					
Aspergillus sp.	—					
Botrytis cinerea					—	
Cephalosporium (2 sp.)	—					—
Cladosporium (4 spp.)		—	—	—	—	—
Cryptosporiopsis sp.				—	—	—
Cylindrocarpon sp.	—		—	—	—	—
Dictyopolyschema pirozynskii			—		—	
Epicoccum sp.	—		—	—	—	—
Graphium sp.						—
Leptographium lunderbergii			—			
Paecilomyces elegans	—		—	—	—	
Paecilomyces farinosus			—			—
Penicillium (3 spp.)	—	—	—	—	—	—
Phoma (2 spp.)	—	—	—	—	—	
Trichoderma (3 spp.)	—	—	—	—	—	—
Truncatella sp.				—		
Ulocladium chartarium	—	—	—			
Verticillium chlamydosporium	—					
Verticillium lecanii		—	—	—	—	—
Verticillium nigrescens				—	—	—
Verticillium nubilum	—	—	—			

Bacterial diversity: Bacteria were most abundant in the surface layers of stumps (15 mm) than deeper, although numbers found deeper within stumps gradually increased with stump age (Fig. 2). Numbers of CFUs isolated within stumps significantly reduced ($P>0.001$) with stump age after year 3 (Fig. 3), irrespective of the position of isolation.

The Shannon-Weaver index indicated that diversity differed significantly between samples taken from nearest the stump surface and those taken from deeper in the stumps (2-sample t-tests; $p<0.01$). No significant difference was found in these samples using Simpson's index. Changes in bacterial diversity measured using the Shannon-Weaver index differed significantly with stump age, showing a quadratic relationship ($r^2 = 0.0519$; $p<0.05$; Fig. 4).

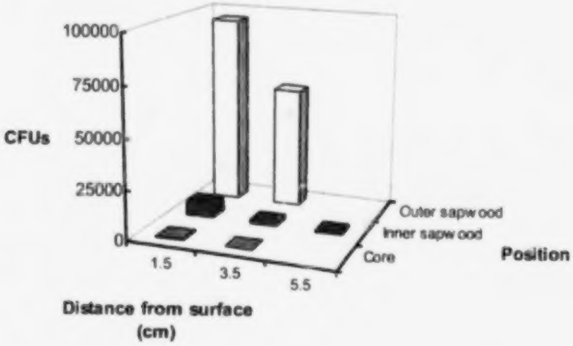

Figure 2. Enumeration of colony-forming bacteria at different sampling positions in Sitka spruce stumps.

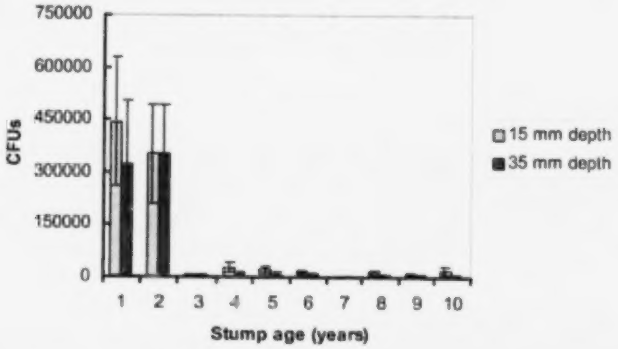

Figure 3. Numbers of colony-forming bacteria (±SEM) found in outer sapwood samples of Sitka spruce stumps. Samples were taken from 15 mm and 35 mm depth below the stump surface. Similar temporal patterns were found for other stump sampling positions.

DISCUSSION

The conifer stump is a complex environment, and numerous microbial interactions occur as the plant tissues die and begin to decay. The results described here demonstrate the high numbers of fungi and culturable bacteria which are present in the niche. It is known that enumeration of bacteria using CFUs as the measure is subject to inaccuracies; recent estimates have suggested that colony counting may under-estimate numbers present by a factor of between 4 and 6 orders of magnitude in oligotrophic systems (Amann and Ludwig 2000). Conifer stumps, with low nutrient availability, may also encourage the development of non-culturable prokaryotes, and the future application of rRNA probes to this niche will increase our understanding of the diversity present. Despite these reservations, the numbers of bacteria present in stumps of up to 2 years in age were clearly extremely high, and the diversity experiments suggest that these organisms make a large contribution to the numbers of species present in this niche.

Diversity of the hymenomycetes increased with stump age over the time observed in these studies; fungi in other phyla, however, showed no pattern of establishment. With the bacteria also, changes in apparent diversity estimated using the Shannon-Weaver index were not clear: the quadratic correlation was weak.

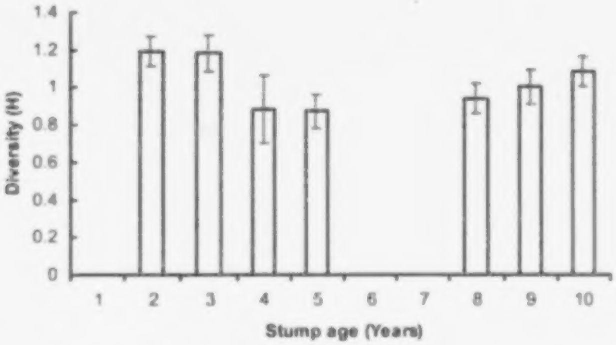

Figure 4. Mean bacterial diversity in Sitka spruce stumps up to 10 years old (±SEM) using Shannon-Weaver diversity index (H). A significant quadratic relationship was found between diversity and stump data ($y=0.015x^2-0.191x+1.490$; $r^2=0.0519$).

Many species of Ascomycota and mitosporic fungi were found at one sampling time only. Species of common moulds, in genera such as *Aspergillus*, *Cladosporium* and *Penicillium* probably occur by chance, utilising free sugars which become available in the upper tissues of the stump. Some of the fungi obtained may also have arisen from spores present in the stump, which germinated on the sugar-rich media used in the culturing process.

No attempt was made to distinguish species of bacteria present in the stumps; diversity indices were calculated based on clearly defined differences in colony morphology. Further distinctions between isolates were made using enzyme activities (Murray and Woodward, this volume), and it was clear that a large number of variants were present within the community of culturable bacteria. Many of the CFUs obtained were able to inhibit the growth of *H. annosum* (Murray and Woodward, this volume) and other decay fungi (Murray 1998) and are likely to influence the succession of micro-organisms occurring in spruce stumps.

The data obtained in this work describe the early stages of stump decay, particularly for the fungi, under conditions present in north-east Scotland. Numbers of species may increase further with time, although as decay fungi become more dominant, they could exclude many other species. The production of antimicrobial metabolites by these fungi (cf Woodward et al. 1993) will have a marked influence the abilities of different fungi or bacteria to establish in the niche in competition with the dominant species. The drop in numbers of bacteria recovered from stumps of greater than 3 years old may reflect the capture of large domains by individual genets of antibiotic-producing hymenomycetes (Woods 1996).

Greater microbial diversity may be detected in a niche using molecular methods, such as direct PCR amplification of ITS regions in extracted DNA, and RFLP analysis or sequencing of the products (Johannesson and Stenlid 1999; Amann and Ludwig 2000). Attempts to use molecular methods to determine the composition of fungal communities in dead wood of Norway spruce were hampered by the inefficiency of standard DNA extraction methods when applied to woody substrates (Johannesson and Stenlid 1999; Vaino and Hantula 2000). With further refinement, it will be possible to enumerate fungi more accurately in the stump, allowing rapid analysis of species composition and populations.

The results presented here demonstrate the high degree of microbial diversity present in stumps of Sitka spruce growing in Scottish plantations. Clearly, if these data are added to those already known for biodiversity in this ecosystem (Humphrey et al. in press), then the true contribution of such plantations to diversity can be better appreciated. Further work is required to determine the full bacterial diversity present in stumps, and to compare microbial diversity in other Scottish forest ecosystems, such as the semi-natural Scots pine forests and the mixed conifer-broadleaf forests found in the locality.

ACKNOWLEDGEMENTS

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BACTERIAL DIVERSITY IN SITKA SPRUCE STUMPS AND THEIR INTERACTIONS WITH DECAY-CAUSING FUNGI

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SUMMARY

Changes in the bacterial community of Sitka spruce stumps between 1 and 10 years old were investigated by isolating from seven positions within the top 55 mm of the stumps, and in vitro interactions between isolates and *Heterobasidion annosum* determined. Distribution of colony-forming units (cfu's) in stumps was affected by both stump age and sampling position. Grouping of cfu's on the basis of 13 phenotypic characteristics, including colony form and colour, Gram stain, heat tolerance, siderophore production and production of various degradation enzymes, identified four groups of bacteria, the proportions of which varied with stump age. Bacterial diversity was higher nearer the stump surface than deeper inside the stump.

Interactions between bacterial isolates and *H. annosum* on agar-based substrates varied depending on the medium used. In spruce wood blocks, the relative timing of bacteria and fungal inoculation had a significant effect on the outcome of the interactions. Co-inoculation of bacteria and *H. annosum* significantly reduced the rate of degradation relative to controls, whereas introducing the bacteria 10 days prior to the fungus generally reduced the degradation rate.

The results are discussed in terms of the possible influence of prokaryotic organisms on the development of decay communities in Sitka spruce stumps.

Keywords: Sitka spruce, bacteria, diversity, *Heterobasidion annosum*, interactions

INTRODUCTION

Studies of microbial succession in conifer stumps have focused on mycelial fungi, although large numbers of yeasts and bacteria are frequently reported in these communities (Kallio 1974; Hallaksela 1977). The role of prokaryotic organisms in decay community development in stumps, however, has received little attention (Aho et al. 1974; Hallaksela 1984), probably due to difficulties in working with bacteria. These organisms are abundant in spruce stumps (Hallaksela 1977; Murray 1998) and undoubtedly influence the succession of organisms occurring, which eventually leads to dominance by hymenomycetes, such as *Heterobasidion* spp. (Holdenrieder and Greig 1998). Changes in bacterial populations have been noted during development of decay communities in both wounded living conifer trees (Kallio 1974; Hallaksela 1984) and stumps (Hallaksela 1977; Murray 1998; Woodward in press), and it has been suggested that prokaryotes may have a fundamental role in the initial development of microbial communities in woody substrates (Shortle et al. 1978; Hallaksela and Salkinoja-Salonen 1992).

This paper describes the diversity of bacterial populations found in stumps of Sitka spruce 1 to 10 years after cutting, and the interactions between selected baterial isolates and *Heterobasidion annosum*.

MATERIALS AND METHODS

Stump material: Bacteria were isolated from thinning stumps produced in routine forest management operations in several mature upland Sitka spruce (*Picea sitchensis*) plantations (planted 1932 - 1967) located within 70 km of Aberdeen. Sites selected had clear records for thinning carried out over a 10 year period. At each site, bacteria were isolated from five randomly selected thinning stumps of 1 - 10 years in age since felling. The stump surface was brushed clean, loose bark removed and the top 100 mm removed with a chain saw. A 75 mm wide section of the disc was removed and transported in a clean plastic bag. Sections were stored at 5°C within 2 h of cutting.

Isolation of Bacteria: Wood tissues (3 cm^3) were removed aseptically from various positions in the discs using a 10 mm diam. drill bit to a depth of 40 mm. Samples were vortex mixed in 10 ml sterile saline solution (0.9% NaCl) and bacteria enumerated by plating 0.1 ml aliquots of a 10^4 dilution onto R2A medium (Reasoner and Geldreich 1985), containing 50 $mg.l^{-1}$ cycloheximide. Cultures were incubated at 20±2°C and colony-forming units (CFU) sub-cultured as required.

Characterisation of bacteria: Four hundred and seventy isolates were characterised on the basis of colony morphology, Gram stain reaction, formation of endospores at 80°C, and the production of enzymes and siderophores (Schwyn and Neilands 1987). Colonies on R2A medium were classified into one of 10 colour categories (white, off-white, cream, pale yellow, orange/brown, pale pink, pink, purple and grey/green). Colony shape and size (very small < 1mm; small 1-2 mm; medium 3-5 mm; large > 5 mm) were determined using well-separated 7 days old colonies. Degradative abilities tested were cellulase (Barlows 1992), 'ligninase' (Gold et al. 1988), amylase, pectinase and chitinase (Page et al. 1982). Catalase activity was tested using 3% H_2O_2 and observing for evolution of O_2 bubbles. Oxidase (as cytochrome C) was detected using Kovacs' reagent.

Based on the different characteristics determined, CFUs were clustered using the Euclidean distance measure and the group average linking method. Gram positive and Gram negative CFUs were analysed separately. Groups were identified from clusters when 75% of the information remained.

Interactions between bacteria and *Heterobasidion annosum*

Interactions on Agar-based media: Petri dish cultures of *H. annosum* were prepared on both sporulation agar (SA) and yeast-peptone-dextrose agar (YDPA; Benko and Highley 1992), co-inoculated with a bacterial isolate (100 tested in total) from stumps of 3, 4, 6 and 10 years old, and incubated in the dark at 20±2°C.

Interactions in Liquid Culture: Fifteen bacteria inhibiting *H. annosum* growth on SA were sub-cultured to 15 ml R2A broth and incubated in the dark at 20±2°C for 72 hr. Following mixing, 5 ml of broth was transferred to a 50 ml jar containing 15 ml Norkrans' liquid medium (NLM: Norkrans 1963). Jars were inoculated with actively growing *H. annosum* mycelium on 2% malt extract agar, sealed and incubated at 20±2°C for 21 days. Cultures were filtered through 2 layers of muslin cloth, washed with 50 ml distilled water and dried to constant weight at 105°C.

Interactions in P. sitchensis Wood Cubes: Cubes (20 mm^3) cut from green *P. sitchensis* timber, were weighed, placed in a 50 ml glass jar and 3 ml tap water added. Jars were sealed and autoclaved at 105 kPa for 1 hr. Ten wood cubes were re-weighed, dried at 105°C to constant weight and re-weighed to obtain an initial dry weight.

Fifteen bacterial isolates showing antagonism against *H. annosum* on SA were sub-cultured into 15 ml R2A broth. *H. annosum* was sub-cultured from MEA to 15 ml NLM, incubated at 25±2°C for 10 days and cultures fragmented using a sterile Ultra Turrax homogeniser set at half-speed.

Autoclaved wood cubes were inoculated with bacteria and fungi simultaneously, or 10 days apart, in all possible combinations, by placing 1 ml of each culture onto the wood. Controls cubes were not inoculated, or inoculated with bacteria or with *H. annosum* only. Jars were sealed using Parafilm M and incubated at 20±2°C for 140 days after inoculation with *H. annosum*. Wood cubes were then removed from the jars, rinsed in distilled water and dried to constant weight at 105°C. Percentage weight loss due to fungal or bacterial activity was calculated.

Statistical Analysis: Results were analysed using the student's t-test, analysis of variance (ANOVA), Duncan's multiple range test and the χ^2 test.

RESULTS

Bacterial isolations from stumps

Types of bacteria, based on characteristics: Proportions of CFUs able to hydrolyse cellulose increased significantly ($P<0.05$) with stump age (Fig. 1a), but no such relationship was found with pectin or starch. No bacteria were able to decolorised Remazol Brilliant blue R. Siderophore production was inversely correlated with stump age (Fig. 1b). The proportion of CFUs with chitinase activity increased significantly with stump age ($P<0.05$), although numbers with this activity were low.

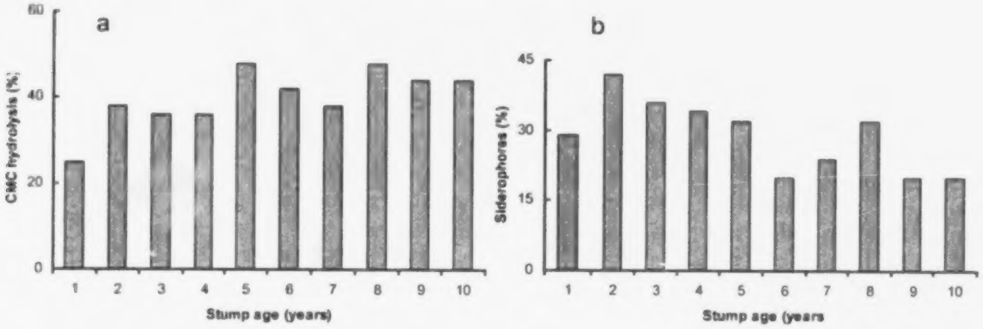

Figure 1. Percentage of bacteria isolated from Sitka spruce stumps of increasing age able to (a) hydrolyse cellulose (as carboxymethylcellulose) or (b) produce siderophores. There was a significant ($P<0.05$) linear relationship, $y=1.636x+30.933$ ($r^2=0.527$), between increasing stump age and proportion of bacteria able to degrade CMC and a significant ($P<0.05$) inverse linear relationship, $y=1.818x+38.933$ ($r^2=0.515$), between increasing stump age and proportion of bacteria producing siderophores.

Euclidean distance measure and the group average linking method identified 3 distinct groups within the Gram negative and 1 group in the Gram positive bacteria. Each group contained at least 5% of the total number of isolates tested. These 'groups' different in abundance with stump age (Fig. 2). Chi-squared tests found significant differences in the frequency of isolation of Group A bacteria ($p<0.01$), Group B ($p<0.05$) and Group C ($p<0.01$) in different stump age groups; Group D bacteria, however, were isolated in similar numbers from all stump ages.

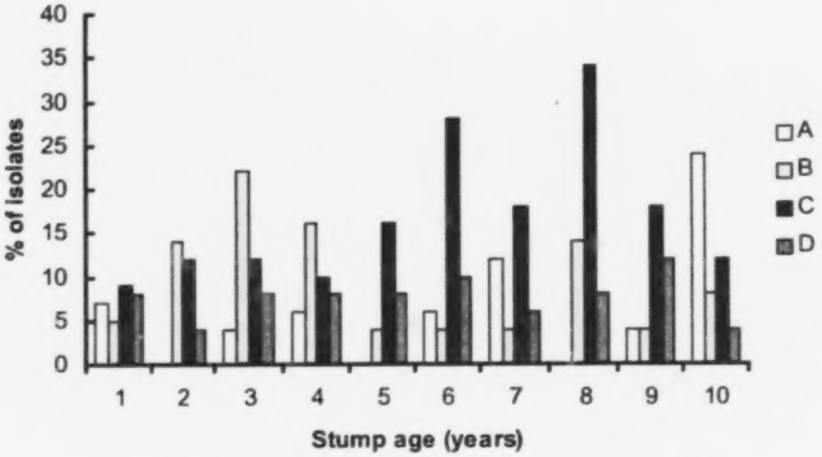

Figure 2. Proportions of bacteria in Groups A, B, C and D found in Sitka spruce stumps of increasing age.

Interactions between bacteria and *Heterobasidion annosum*

Interactions in and liquid agar media: Thirty percent of bacteria tested were found to inhibit growth of *H. annosum* on SA and on YDPA, although only 10% of isolates caused inhibition on both media. There was no significant correlation between the ability of bacterial isolates to inhibit growth of *H. annosum* on SA or YDPA ($\chi^2_{[1]} = 0.226$). In addition, 3% of isolates promoted growth of *H. annosum* on YDPA.

There was no correlation between proportions of bacteria inhibiting *H. annosum* on SA and age of the stumps from which the bacteria were isolated ($\chi^2_{[3]} = 0.952$). In contrast, inhibition on YDPA was significantly correlated with stump age ($P<0.001$; $\chi^2_{[3]} = 24.571$). More inhibitory isolates were found in 3 and 10 year old than in 4 or 6 year old stumps.

Twelve bacteria causing inhibition of *H. annosum* on SA also had a significant inhibitory effect in NLM.

Interactions in Wood Cubes: Inoculation of wood cubes with bacteria alone caused no significant weight loss over 140 days ($P>0.05$; Fig. 3). *H. annosum* alone caused a 2.04% weight loss from cubes, a significant change (t-test; $P<0.05$) compared with controls. Inoculation of wood cubes with bacteria 10 days after inoculation with *H. annosum* resulted in a significant increase ($P<0.001$) in weight loss compared to inoculation with *H. annosum* alone. In contrast, weight loss was significantly lower ($P<0.05$) in cubes simultaneously inoculated with bacteria and *H. annosum*, in comparison with *H. annosum* controls. In wood cubes inoculated with bacteria 10 days before with *H. annosum*, no significant weight loss ($P>0.05$) occurred compared with cubes inoculated with *H. annosum* alone.

DISCUSSION

This work indicates the potential importance of bacteria in microbial community development in Sitka spruce stumps. Enumeration suggested that bacteria are present in large numbers during the first two years in the upper layers of the stump (Murray 1998; Woodward in press), and that the culturable population decreases markedly after this time, possibly due to the establishment of, and antibiotic production by, hymenomycete decay fungi in the substrate by this time.

The abilities of the bacteria to degrade different substrates did not correlate strongly with stump age. Lignolytic activity, based on the use of the lignin-like substrate Remazol brilliant blue R, was absent in the isolates tested, although certain bacteria are capable of degrading this substrate (Singh and Butcher 1991). Numbers of isolates with cellulase activity, however, did increase with stump age, and was also correlated with wood moisture content (Murray 1998). Whether this pattern resulted from selection for cellulolytic bacteria under higher moisture conditions, or the higher moisture conditions followed the degradation of cellulose was not determined. Pectinase activity was detected in 3.2% of bacterial isolates from the stumps, although the specific substrate present can be of great importance in inducing this group of enzymes (Dunleavy *et al.* 1973).

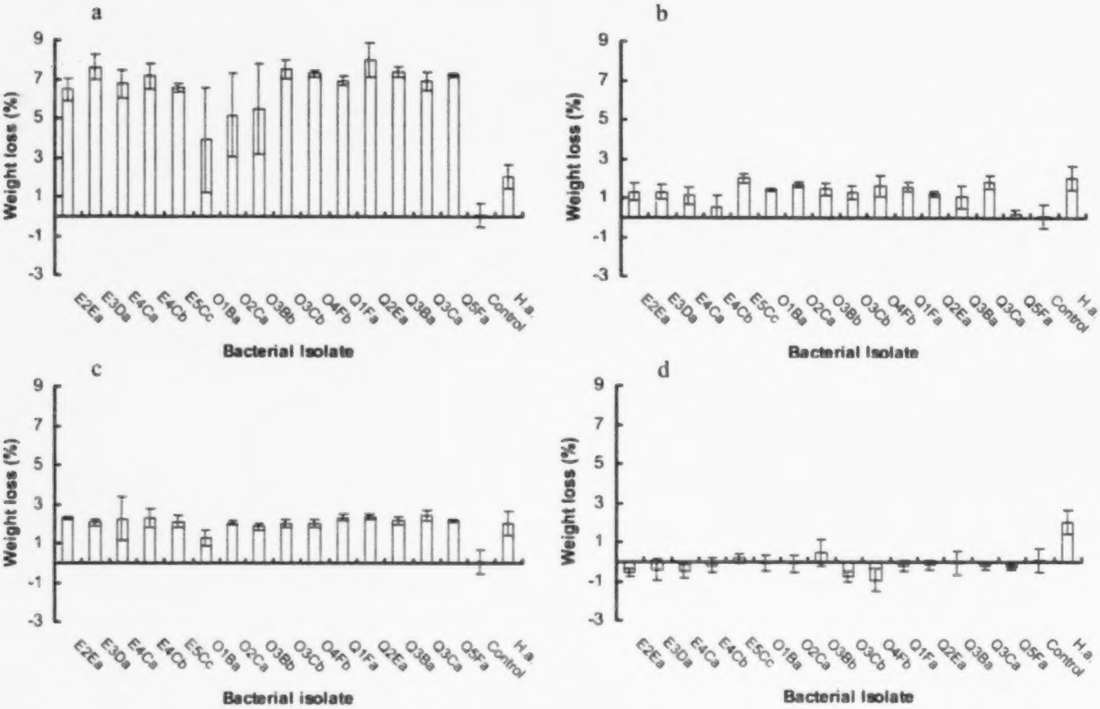

Figure 3. Weight loss of Sitka spruce wood cubes 140 days after inoculation with *Heterobasidion annosum* in combination with 15 bacterial isolates showing inhibition of *H. annosum* on SA. Blocks were inoculated with (a) *H. annosum* followed 10 days later by bacteria; (b) *H. annosum* and bacteria simultaneously; (c) bacteria followed 10 days later by *H. annosum*; (d) bacteria alone. Bars for controls (no inoculation) and *H. annosum* inoculation alone (Ha) are included on each graph. Values represent means of three replicates ± SEM.

Amylase production may also be important for stump bacteria as starch is the major storage polysaccharide found in wood parenchyma cells (Zabel and Morrel 1992). Approximately 8% of bacteria isolated from stumps were amylase positive, and proportions varied with stump age, with high numbers in stumps of 1 and 9 years old. Chitin hydrolysis has a role in the biological control of fungi by bacteria, acting on the fungal cell wall (Whipps 1997). A significant positive correlation was found between the proportion of stump bacteria with chitinase activity and stump age, but the actual numbers of individual isolates found to produce chitinase, 2.1% in total, was small.

Under the inducing conditions found in the stump, very different from those in axenic cultures, the production of requisite degradative enzymes may occur. However, it is likely that the use of specific substrates as sole carbon sources in *in vitro* tests will induce the production of the enzymes required to degrade that substrate, if the organism is competent.

Siderophores were produced by 29.3% of bacteria tested, but decreased in bacteria from older stumps. There were also significant correlations with position in the stump, and with heartwood vs sapwood isolates (Murray 1998). Production of chelating agents by bacteria commonly occurs under iron deficient conditions, and has been implicated in competition between bacteria and fine root pathogens (Whipps 1997). In contrast, germination and growth of some fungi, including *H. annosum*, can be markedly enhanced in the presence of siderophores (Blakeman 1982). The chelating ability of bacteria found in young stumps may reflect a requirement to sequester nutrients present in low concentrations at this early stage in the degradation process.

Four groups of bacteria were identified using cluster analysis based on 13 characteristics. This method was suitable for a general discrimination between the bacteria, and population changes in the stumps. Greater numbers of characteristics are required to give accurate taxonomic discrimination (Goor *et al.* 1990). Proportions of the three groups of Gram negative bacteria varied significantly with stump age, whereas the proportions of the single group of Gram positive isolates remained similar in all stump ages. Few studies have been published on bacterial successions in decaying woody debris in the forest.

An appreciation of the numerous interactions that occur between microbial populations in a natural community is essential to an understanding of the functioning of that community (McInerney 1986; Rayner and Boddy 1988). Although there are difficulties inherent in predicting *in vivo* interactions based on *in vitro* assays, such tests provide rapid, simple and inexpensive methods for screening interactions between micro-organisms. There is a tendency for *in vitro* tests to favour species producing of siderophores or antifungal metabolites (Nicolotti and Varese 1996; Dumas 1992). Variations in the proportions of bacteria inhibiting *H. annosum* with medium used to test the interaction clearly illustrate the difficulties in finding suitable media for *in vitro* studies to mimic natural environments.

Liquid media enable immediate and intimate contact between interacting micro-organisms, and differing responses were found with the 15 bacteria which inhibited *H. annosum* on SA, when tested in NLM. Greater access to available nutrients from throughout the medium may increase the importance of competition as a mechanism of antagonism in this environment. Three isolates active against *H. annosum* in SA had little effect on the fungus in NLM, however, possibly resulting from their relative abilities to compete with *H. annosum* in an environment where diffusion of metabolites is relatively easy compared with solidified substrates (Begon *et al.* 1990). In such a situation, the species with the greater initial inoculum size may inhibit the growth of the competitor.

Weight loss in wood cubes inoculated with *H. annosum* was only 2% over the 140 days of incubation, and the effects of co-inoculation with bacteria varied, particularly in relation to the relative timings of the fungal and bacterial inoculations. Similar effects have been noted previously (Hulme and Shields 1972). However, both Henningsson (1967) and Greaves (1970) found bacteria inhibited decay when inoculated into wood between 2 and 21 days before inoculation with fungi. No attempt was made in the current work to re-isolate the bacteria from the wood cubes after incubation, and the lack of any inhibitory effect may have resulted from a failure of the bacteria to establish. Inoculation with bacteria 10 days after *H. annosum*, however, resulted in weight losses over 200% greater than in control cubes. Similar enhancement of decay has been reported previously in tests using bacteria and wood decay fungi (Henningsson 1967; Blanchette and Shaw 1978), and may result in part from an increased availability of certain nutrients produced by the bacteria. Many decay fungi require thiamine and biotin in the external environment, and bacteria may provide these vitamins (Alexander 1977). Fungal cellulase activity also may be enhanced in the presence of bacteria, as the prokaryotes utilise carbohydrate breakdown products which could repress enzyme production (Henningsson 1967; Greaves 1970; Shortle *et al.* 1978).

Given the recent reports citing the detection using molecular methods of high numbers of species of unculturable prokaryotic organisms in many environments (Amann and Luwig 2000), it is likely that tree stumps will also contain far more bacteria than recorded in the work described here. It is also probable that the unculturable prokaryotes present within the niche alter their immediate micro-environment through the production of enzymes and different secondary metabolites. The results presented here and elsewhere in this volume (Woodward in press), however, emphasize the potential for bacteria to exert considerable influence on the development of the decay community in spruce stumps.

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SWISS-STONE PINE TREES AND SPRUCE STUMPS MAY REPRESENT THE PRIMARY HABITAT FOR *HETEROBASIDION ANNOSUM SENSU STRICTO* IN WESTERN ITALIAN ALPS

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SUMMARY

In the Western Italian Alps, all three species of the forest pathogen *Heterobasidion annosum* coll. are present, and may even coexist in the same stand. While the presence of *H. parviporum* and *H. abietinum* can be easily correlated to the presence of their respective primary hosts, spruce and fir, the host/niche occupied by *H. annosum sensu stricto* (s.s.) still remains unclear. Although Scots pine, a major host for this fungal species in other parts of Europe, is abundant in the region, little or no evidence of disease caused by *H. annosum* is visible in this tree species. An analysis of *H. annosum* coll. species was performed in two natural mixed conifer forests using traditional isolation techniques and a novel direct molecular diagnosis from wood. In site 1 (Cogne, Aosta Valley), a subalpine stand of mixed spruce, larch, and Swiss-stone pine (*Pinus cembra* L.), 16 naturally infected spruces and larches only yielded *H. parviporum* isolates, while a Swiss-stone pine was extensively colonized by both *H. parviporum* and *H. annosum* s.s. In site 2 (Charvensod, Aosta Valley), a stand very similar to the above one, an analysis of 15 spruce stumps yielded both *H. parviporum* and *H. annosum* s.s. isolates. At both sites Scots pine was absent. These results suggest that 1) Swiss-stone pines and larches may be infected by the locally predominant *H. annosum* species (i.e. *H. parviporum*); 2) Swiss-stone pine may be a primary host for *H. annosum* s.s.; and 3) spruce stumps may be colonized by both *Heterobasidion* species and may thus offer a suitable habitat for the survival of *H. annosum* s.s. in the region. To our knowledge, this is the first report of *Heterobasidion annosum* on native European *Pinus cembra* and of an adult pine tree to be contemporary colonized by isolates belonging to different species of *H. annosum*.

Keywords: *Heterobasidion annosum*, *Pinus cembra*, *Larix decidua*, ISGs, ecology

INTRODUCTION

Subalpine forests are, despite their low tree species diversity, structurally and spatially highly heterogeneous. These forests consist of a mosaic of stands, tree groups, single trees and glades. From the boundaries of subalpine ecotones up to the timberline, trees are more and more influenced and controlled by a range of environmental factors; the temperature is usually identified as the main one (Holtmeier 1993).

In the Western Italian Alps (WIA), fir (*Abies alba* Miller) and especially spruce forests are severely affected by the root and butt rot agent *Heterobasidion annosum* (Fr.) Bref. coll. (Anselmi and Minerbi 1989, Capretti 1998, Cellerino et al. 1998). While in other parts of the world the damages caused by *Heterobasidion* tend to be smaller at higher altitudes (Korhonen and Stenlid 1998), in WIA they are locally relevant at high elevations (1800-2300 m a.s.l) as well (Gonthier 2001). Unpublished data point out that some subalpine forests in Aosta Valley (WIA) are infected up to 95% of the trees. No more information are available about the epidemiology of *H. annosum* coll. in subalpine forests.

Norway spruce (*Picea abies* (L.) Karsten), European larch (*Larix decidua* Miller) and Swiss Stone pine (*Pinus cembra* L.) are the main components of the subalpine forests in WIA. Among these, larch and especially spruce are well documented *H. annosum* coll. hosts (Korhonen and Stenlid 1998). Spruce is the main host for *H. parviporum* in Europe (Korhonen et al. 1998) and strong host specificity of this fungus vs spruce has been recently confirmed in pure and mixed forests growing at lower elevations in WIA (Gonthier et al. 2001; Gonthier 2001). Besides, both *H. annosum sensu stricto* (s.s.) and *H. abietinum* have been occasionally found on spruce, mostly in Northern and Southern Europe respectively (Korhonen and Piri 1994, Vasiliauskas and Stenlid, 1998, Capretti et al. 1994). *Heterobasidion annosum* s.s. and *H. abietinum* are known to infect, in the same areas respectively, *L. decidua* too (Stenlid 1987, Capretti et al. 1994, Vollbrecht et al. 1995), even if that coniferous species shouldn't be considered as a primary host for *H. annosum* coll. (Korhonen and Stenlid 1998). Little is known about the incidence of the disease and the behavior of *Heterobasidion* on the Swiss Stone pine, since this tree species is rarely felled in WIA. The fungus has been reported on *P. cembra* only once, right in the WIA (Nicolotti et al. 1999), and it was typed as *H. parviporum*. The finding of *H. annosum* on *P. cembra* is an obvious cause for concern because 1) it is generally believed that this tree species is resistant to most decay agents; 2) the pathogen was causing an extensive butt rot in the infected tree, whereas it is normally reported as root rot agent vs other pine species; and 3) *H. parviporum* is generally considered a pathogen of spruce trees and not pines.

Recent completed studies on the epidemiology of *H. annosum* coll. in WIA show that in the region the scenario is complex (Gonthier et al. 2001, Gonthier 2001). Synthetically 1) all the three species of *H. annosum* are present; 2) they may coexist in the same stand; 3) strong host specificity is showed by *H. parviporum* and *H. abietinum* vs their preferential hosts. While the presence of *H. parviporum* and *H. abietinum* can be easily correlated to the presence of their respective preferential hosts, the host/niche occupied by *H. annosum* s.s. in that area still remain unclear. This fungus has been found in forests either with or without Scots pine (*Pinus sylvestris* L.), one of the major host for this fungus in other parts of Europe (Korhonen et al. 1998). In WIA, even in stands where both *H. annosum* s.s and its putative preferential host are presents, no evidence of disease is visible in this tree species. Two different, but not mutually exclusive, hypotheses can explain the presence of *H. annosum* s.s. in the WIA. 1) Scots pines are infected but largely asymptomatic; and/or 2) *H. annosum* s.s. has adapted to different hosts and/or niches.

The aims of our study in mixed spruce, larch and Swiss stone pine forests were 1) to describe the symptoms caused by *H. annosum* on *P. cembra*; 2) to identify the *Heterobasidion* species present in the infected pine and in adjacent trees, *iii*)- to investigate the patterns of colonization of individual fungal genotypes. Besides, an adjunctive objective was *iv*) to evaluate the role of other hosts/niches in the survival of *H. annosum* s.s. in the region.

MATERIALS AND METHODS

Sites and stand descriptions

Two experimental plots were located in two naturally regenerated subalpine forests, which lay one another in parallel mountain slopes, in Aosta Valley (WIA). Both forests face North in valleys with an alpine sublitoral climate (mean annual rainfall 750 mm). The sites are classified as *Picetum subalpinum* and the soils as Ochrepts/Umbrepts. The two forests are very similar, both as regards the tree species composition and the structural aspects, and they comprise several large even-aged groups of trees, and as a whole is uneven-aged. Spruce is the dominant species (about 60% of the canopy); larch and Swiss stone pine represent about 20% of the total number of trees. Some silvicultural characteristics of the investigated forests are shown in Table 1. Regeneration of *P. cembra* is present in groups, but the adult trees have been found to be coetaneous, ranging between 85 and 90 years of age. The dominant larches were about twice as old, while spruce trees belonged to different age classes, indicating a continuous regeneration of this species through time.

Table 1. Characteristics of the forests investigated.

Forests	Elevation (m. a.s.l.)	Spruce % [butt rot incidence%][1]	Stone pine % [butt rot incidence%][1]	Larch % [butt rot incidence%][1]	Basal area (m^2 ha^{-1})	Density (trees ha^{-1})	Mean DBH[2] (cm)	DH[3] (m)
Cogne (site 1)	1,800-2,050	60 [90]	15 [?]	25 [30]	25.48	512	25	26
Charvensod (site 2)	1,800-1,900	60 [50]	20 [?]	20 [10]	26.31	400	29	24

[1] This estimate was based on the frequency of stumps displaying the typical laminated white rot caused by *H. annosum* coll. (following thinning in 1997-1999)

[2] Diameter at breast height

[3] Dominant height

In site 1 we studied the symptoms caused by *H. annosum* coll. on *P. cembra* and the patterns of colonization of fungal isolates present in the pine. At this site we also investigated the frequency of the disease in the surrounding trees, and the spatial distribution of individual *H. annosum* coll. genotypes in spruce and larch trees - or just felled secondarily infected stumps - growing within 30 m from the infected pine.

In site 2 we studied the frequency of the different species of *H. annosum* coll. in spruce stumps. That epidemiological information, together with data obtained in the same stand typing spores collected by the wood disk exposure method (Gonthier et al. 2001), could be useful to understand some ecological behavior of *H. annosum* s.s. in WIA.

Site 1: investigations and sample collection from the infected Swiss stone pine tree and from the surrounding stumps and trees

The infected Swiss stone pine tree, growing at 1900 m a.s.l,. was cut in May 1998 and it showed an extensive butt rot in the stem section. The tree, growing closed to an old larch, was 14 m tall and its DBH was 34 cm. Annual rings which were visible (not decayed) were counted and measured both upstream and downstream. To assess the probable age of the tree and if the fungal infection would have cause a reduction in tree growth, two cores were extracted by an increment borer upstream and downstream at 40 cm aboveground from each of 10 healthy *P. cembra* trees growing in the stand. Annual ring values of the infected tree and the healthy ones were then compared.

The symptoms were carefully described and, to assess the extent of the decay inside the tree, the trunk was consecutively cut into 50 cm logs. A longitudinally cut was performed in the last log in which discoloration was noticed. From each stem log, a 2 cm thick disk was taken from the major section for isolations.

The complete root system was excavated to check for the occurrence of root grafts between the infected pine and the surrounding trees and stumps. All the woody roots, down to a diameter of 0.5 cm, were measured (length) and consecutively numbered; root contacts were marked as well. Roots were sampled by excising transversal disks. Where root contacts were observed, samples were also taken from roots of surrounding trees/stumps. The *P. cembra* stump, including about 1.5 m of all its main roots, was dug out, transported to the laboratory, and consecutively cut by a circular saw into 3-4 cm thick disks.

All trees and stumps (left in 1997 after a thinning) with diameter over 4 cm, growing in a range of 30 m from the infected pine, were carefully mapped. The DBH was measured for trees and estimate for stumps. Each tree was sampled by extracting 2-3 cores from the collar zone and each stump by cutting off its top portion to obtain a 6-7 cm-thick disk near the root collar. Basidiocarps were also collected and stored at 5°C.

Disks and cores were sprayed with a benomyl solution (0.010 g benomyl, 500 µl methanol, 1 l sterile water) and incubated, according to their size, in plastic bags or in Petri dishes (15 cm diam.), containing damped filter paper, for about 10 days at room temperature. Colonies of *H. annosum* were recognized by their *Spiniger* stage. In the Swiss stone pine, any distinct area bordered by interaction zones on the disks was identified and numbered. Corresponding areas on the following disks were numbered according to previous disks. Isolations were made from any area on PCNB-based selective medium (Kuhlman and Hendrix, 1962). Isolates from basidiocarps were obtained from the context.

The larch tree, growing closed to the pine (about 0.4 m to each other), was felled down. The stem section showed a typical *Heterobasidion* discoloration, and a disk was taken. As it was impossible to isolate the fungus, a wood block (1.5 x 1.5 x 1.5 cm), including the discolored area, was cut and frozen at -20°C before DNA extraction.

Site 2: sampling of spruce stumps for species composition assessment

Fifteen randomly selected healthy spruce stumps were sampled in July 1998 (Gonthier 2001). The trees were cut 1 yr before. A wood disk (2-3 cm thick) was taken about 15 cm below the top surface of each stump. Stumps showing visible wood discoloration at the collar, where discarded, to avoid isolation of colonies spreading from roots. The treatment of samples, the incubation period and the isolation techniques were as above.

Mitochondrial and nuclear typing for the assessment of *H. annosum* species and somatic incompatibility tests.

All isolates were checked for the presence/absence of clamp connections, that would indicate either heterokaryon or homokaryon, respectively.

The following methods were employed to classify the isolates. First, a taxon-specific competitive-priming (TSCP-PCR) (Garbelotto et al. 1996) in the mt LrRNA gene, as described by Gonthier et al. (2001). To type all three European species of *H. annosum* by a single PCR amplification and gel, the method was modified as follows. A mix of four primers (MLS, MLF, Mito 5 and Mito 7) was used for DNA amplifications, and PCR products were electrophorezed in 2.5% Metaphor agarose gels in 1 X TBE at 3 V cm^{-1} for 3 hrs. Second, a PCR RFLP on ITS to distinguish between *H. parviporum* and *H. annosum* s.s., as published by Gonthier et al. (2001), and to verify the presence of hybrid heterokaryons. Results of molecular typing, both from mitochondrial and nuclear markers, were compared to check for the occurrence of nuclear-mitochondrial chimeras among species. Third, sexual compatibility tests with homokaryon testers of the three species (T4, T5, T6 - kindly provided by Dr. Capretti - , A2r, A27r and A66r) as described by Stenlid and Karlsson (1991). The Buller phenomenon was used to identify heterokayon isolates, and in that case the occurrence of clamp connections was verified in the tester thallus, at least 3 cm from the interaction zone.

Direct DNA extraction from the wood of the larch was performed as follows. Sawdust from collected wood was obtained by drilling in the collected wood. The sawdust was placed in a 1.5 ml Eppendorf tube and DNA was extracted from the sawdust by adding an equal volume of 24:24:1 phenol:chloroform:isoamyl alcohol and vortexing vigorously for 2 minutes. After 15 min centrifugation at 13000 rpm, the supernatant was collected and the extraction was completed using the GeneClean II Kit (QBiogene), following the manufacturer's instructions. DNA was diluted 1:100 in PCR water and a nested PCR approach was utilized to obtain DNA amplification of *Heterobasidion*. It should be noted that the pathogen was no longer culturable and expected to be present in very small titer. A first round of PCR (using parameters described by Garbelotto et al. 1996) was performed with primers ITS-1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). The PCR product was then diluted 1:100 in PCR water and a second PCR amplification was performed using two internal primers (ITS1 and ITS2) (White et al. 1990). Sequences of the complimentary strands of the PCR product were obtained using an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA). Sequence alignments were obtained by using Sequencher software (GeneCodes) and optimized by manual alignment. Using the consensus sequences, a BLAST search of the GenBank database (NCBI) was performed.

To study the presence and the patterns of colonization of *H. annosum* genotypes, all isolates from Cogne, and belonging to either of *H. annosum* species, were paired at least twice in all possible combinations according to the method described by Stenlid and Karlsson (1991).

RESULTS

The infected Swiss stone pine tree was apparently in good health conditions. The color of the foliage and the crown density were similar to those of uninfected *P. cembra* growing in the stand. Seventy-five annual rings were visible on the pine stump, but it is likely that the tree was between 88 and 90 yrs old (age of surrounding *P. cembra* trees). A great reduction in ring size was observed in the last 25 yrs, both vs rings of previous yrs in the same tree (0.72 vs 2.34 mm/yr) and vs the last 25 annual rings from surrounding *P. cembra* trees (1.47 ± 0.02 mm/yr).

Stem decay at breast height occupied about 29% of the stem section and it was confined to the heartwood. The butt rot was fibrous, soft, pale brown to dark brown and appears quite dry. Peripheral areas of the decay were stained either brown or lilac and were bordered bluish or grey. A hollow was noticed from the collar zone up to 2 m of height, while the fungus was isolated from disks taken up to 4 m above the collar.

Decay was also present in the central cylinder of all the seven tree's main roots, down to a diameter of 7 cm. Wood rot and staining appeared similar to those found in the stem. The fungus has also been isolated from 30% of fine roots (0.5-7 cm), that did not show any visible symptom of decay. In that case *H. annosum* was present on the whole section of the sampled disks. No *H. annosum* mycelium was observed between the bark and the wood.

All sampled disks yielded heterokaryon isolates. Isolates from the stem and from one root were typed as *H. parviporum* and they all belonged to the same genotype, whereas the other six main roots were colonized by a single *H. annosum* s.s. genet. In the stump, a distinct dark line of about 2 mm width was present between wood occupied by either of the two fungal individuals. Root contacts were detected between infected fine roots of the pine and infected roots of two closed spruce stumps. Both stumps yielded *H. parviporum* isolates, and one of these was from the same genotype of the pine, suggesting a direct tree-to-tree spread of the fungus.

Of the 35 sampled surrounding trees/stumps (27 spruce, 4 larch, 4 Swiss stone pine), the fungus was isolated from 17 (49%). No isolates were obtained from other surrounding Swiss stone pine trees. The fungus was present in 17 out of 27 sampled spruce tree/stumps (63% incidence) and it was detected also in the larch close to the infected pine. A single *Heterobasidion* basidiocarp was found in a decay pocket of a spruce stump. All isolates possessed clamp connections, all were typed as *H. parviporum*, and each tree/stump, except for the *P. cembra* tree, was colonized by a single *Heterobasidion* individual. The BLAST search resulted in a perfect match between the isolate amplified from larch wood and *H. parviporum*. Among the *H. parviporum* isolates, 14 genets were identified, and these occupied 18 trees (17 spruces and the infected pine) (average of 1.29 tree/stump per genet). The largest genet occupied three trees within a 7 m distance, while other two smaller genets occupied two trees each. Eleven genets (61.1%) were detected in only a single tree. A single *H. annosum* s.s. genotype was present, and it was confined to the Swiss stone pine tree.

Twenty-five *H. annosum* coll. isolates were collected from spruce stumps in Charvensod, and all of them came from the sapwood: 11 (46%) were typed as *H. parviporum* and 13 (54%) as *H. annosum* s.s. Three stumps were colonized by isolates of both species of the fungus, and homokaryotic strains were more frequent than heterokaryotic ones.

Nuclear PCR-RFLP typing indicated the absence of both heterokaryon hybrids and nuclear-mitochondrial chimeras.

DISCUSSION

Although *H. annosum* coll. has already been reported on pine species of the Section *Cembra* (Debazac 1977) before (Kolomiets and Bogdanova 1992, Aref'ev 1991,Darozhkin and Fedarau 1976, Negrutskii 1963), all such reports were from Eastern Europe and Siberia. These areas are well outside the natural range of *P. cembra* L., but correspond to the natural range of the closely related *P. sibirica* Du Tour (Tutin et al. 1993). While we cannot exclude that the pine species in those reports may have been *P. cembra* that had been planted outside its natural range, it is more likely that those reports referred to *P. sibirica* (Korhonen, personal communication). To our knowledge this is the first report of *H. annosum* on *P. cembra* L., at least in the natural range of this pine species.

Heterobasidion spp. are generally reported to kill pine trees, by infecting the cambium and then spreading into the root sapwood (Korhonen and Stenlid 1998). The infected Swiss stone pine examined in this study was apparently symptomless and displayed almost exclusively an internal decay. This unusual characteristics for a pine has already been reported about *Heterobasidion* infections right on trees of the closely related *P. sibirica*, suggesting that these two pine species are mostly susceptible of butt rot instead of root rot. Internal decay was also observed in the root cylinder, often just limited to the heartwood portion of the root. Internal decay of the roots and stem results in a significant loss of structural integrity, and may also, when a large section of the functional sapwood is colonized, lead to a physiological loss of vigor.

Both *H. parviporum* and *H. annosum* s.s. were present in the Swiss stone pine tree, causing a similar heart rot whether in the roots or in the stem. Isolates collected from *P. sibirica* in Southern Finland by Prof. Laine were typed as *H. parviporum* (Korhonen, personal communication). In white fir stands in the United States, multiple *H. annosum* genets were commonly found in living trees (Garbelotto et al. 1999), but the simultaneous presence in single trees of strains belonging to different species of *Heterobasidion* have been reported, as far as we know, only twice, and those reports referred to *P. abies* (Vasiliauskas et al. 1998, C. Delatour, unpublished, in Delatour 1998). Therefore, this is the first report of both pathogen species in a single live pine tree. The presence of both fungal species may indicate that host specificity for these pathogens is not strict, but may be regulated by ecological conditions and site history. In our stand, *H. parviporum* was by far the predominant species, as exemplified by the observation that about 60% of spruces were infected by this fungal pathogen. It has already been shown that the predominance of a pathogen species in a host may result in high percentages of that species in adjacent hosts that may not be generally considered as common hosts for that pathogen (Capretti et al. 1994). This phenomena was verified while studying the Swiss stone pine, as we assessed a secondary vegetative spread of a *H. parviporum* isolate from a spruce to the pine (or vice versa). The same could be also true for the infected larch, as reported by Capretti et al. (1994) about infections of this host in areas characterized by high *H. abietinum* inoculum density. To our knowledge, this is the first report of a *L. decidua* tree to be infected by *H. parviporum*. *Heterobasidion* infections on larches were previously reported as caused either by *H. abietinum* or by *H. annosum* s.s., mostly in Southern and Northern Europe, respectively (Stenlid 1987, Capretti et al. 1994, Vollbrecht et al. 1995).

In the stand at Cogne, the most common host for *H. annosum* s.s., Scots pine, is absent. In general, *Pinus sylvestris* and *P. cembra* do not coexist in the same sites in the Aosta valley and adjoining areas. *Pinus sylvestris*, in fact, is virtually absent at higher elevations, where *P. cembra* grows. In that stand, we found a single *H. annosum* s.s. genet, and it was exclusively associated to the Swiss stone pine tree, while all surrounding sampled trees were infected by *H. parviporum*. This finding suggests that Swiss stone pine is virtually a primary host for *H. annosum* s.s.

Both *H. parviporum* and *H. annosum* s.s. isolates have been found in the forest of Charvensod, and they may even coexist in the same stump. Data on the aiborne inoculum composition of *Heterobasidion* species in that forest have been recently published (Gonthier et al. 2001): 99% of spores were from *H. parviporum* and only 1% of spores were from *H. annosum* s.s. Although further samplings are in need to correctly assess the relative abundance of the two species both in spruce trees and in spruce stumps, this study show a quantitative equilibrium between *H. parviporum* and *H. annosum* s.s. inside primarily infected spruce stumps (46% vs 54%). Results indicate that *H. parviporum* is almost the only species present in the air and thus able to infect stump surfaces. We suppose that large fruit body production and the subsequent massive release of air spora normally occur after colonization of large volumes of substrate, and thus, as found in Cogne, spruce trees are mostly infected by *H. parviporum*. The significant presence of *H. annosum* s.s. inside spruce stumps may be explained by a particular ecological fitness of this pathogen vs this substrate. *H. annosum* s.s. has already been reported to display a saprotrophic degradating ability greater than *H. parviporum* in spruce wood (Daniel et al. 1998). Thus, spruce stumps may represent, in areas were *P. sylvestris* is absent or resistant, an alternate and suitable habitat for the survival of *H. annosum* s.s.

Heterobasidion parviporum and *H. annosum* s.s. co-exist both in spruce stands (Gonthier et al. 2001) and in mixed forests growing at high elevations in the WIA. We show in this study that at least two niches are simultaneously shared by both species in subalpine forests. This finding may have some important implications in the population biology of this fungus, because of the essential role played by true niche overlaps in enhancing the potential for hybridization. Nevertheless, neither heterokaryon hybrids nor nuclear-mitochondrial chimeras between *Heterobasidion parviporum* and *H. annosum* s.s. were found in this study. Absence of inter-group gene flow between the two species has already been reported in the WIA (Gonthier et al. 2001). This finding supports the hypothesis that strong genetic barriers reinforced by negative selection on hybrids are the main factors involved in the lack of hybridization between the two populations in the region.

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RELATIONSHIP BETWEEN SOIL FACTORS, ROOT INFECTION BY *COLLYBIA FUSIPES* AND TREE HEALTH IN *QUERCUS ROBUR* AND *Q. RUBRA*

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SUMMARY

A method of rating of *C. fusipes* root infection severity was used in 2000 in two mature pedonculate oak stands to investigate the relationship between *C. fusipes* infection severity, degree of oak decline and soil factors. This method was also used in young and mature red oak stands, successively in 1995 and in 2001, to investigate the effect of soil factors, such as water logging, and the effects of tree vigour, such as concurrence between trees, on the infection induction and evolution. Thus, four plots in France were selected with a minimum of 51 studied trees on each site. The results clearly showed that pedonculate oaks were more infected by *C. fusipes* when roots were less submitted to water logging and that their proportion of declining increased when they were more infected by the parasite. The level of decline was also influenced by the depth of a gravel layer able to reduce the vertical growth of roots, but *C. fusipes* as been shown to play the major role in the decline. Concerning the work on red oaks, water logging was also shown to negatively influence the infection induction and its evolution. The evolution of infections did not appear to depend on the age of trees or on their initial degree of infection. The period required for an healthy red oak to become heavily infected by *C. fusipes* was computed to be of about 30 years. It was also observed that a moderate or heavy degree of infection could be related to a reduction of tree radial growth. *C. fusipes* appeared to be at the origin of growth loss.

Keywords: *Collybia fusipes*, root rot, oak decline, soil factors

INTRODUCTION

The European oak forest has undergone several diebacks in the last century (Delatour 1983). Around 1980 field studies have mentioned that the primary parasite *C. fusipes* was involved in the decline of pedonculate oak (*Q. robur*), in particular in soils not submitted to water logging (Delatour and Guillaumin 1984; Guillaumin et al. 1985). Presence of *C. fusipes* was also reported in red *oak (Q. rubra*) stands in association with problems of growth. Artificial inoculations have confirmed that *C. fusipes* is a primary parasite and that vigorous seedlings of different oak species are susceptible to *C. fusipes.* Moreover, the parasite was more aggressive on red oak than on pedonculate oak (Marçais and Caël 2000). *C. fusipes* appeared to be a slow parasite, as two years after seedlings had been inoculated, no mortality could be observed as would have occurred with *Armillaria mellea* or *A. ostoyae* on young pine seedlings (Rishbeth 1982). However, the period required for a mature oak to become heavily infected remained unknown. *C. fusipes* is not considered as a weakness parasite, as artificial inoculations evidenced that seedlings defoliated for two years did not show an increased susceptibility to the parasite (Marçais and Delatour 1996). Furthermore, it was observed in a field study that *C. fusipes* did not develop preferentially on trees not favoured by selective logging (Piou et al. 2001). This parasite induces typical orange lesions on large roots and can cause a drastic destruction of the root system. Infected pedonculate oaks, located in sandy soils with presence of a gravel layer, are more declining. Such soils are known to be unfavourable to *Q. robur* and we do not know whether *C. fusipes* was the cause of decline or whether soil conditions are involved in decline. A method allowing a good estimation of root infection severity of a mature tree was established by Marçais et al. (1999). Using this method, we were able to investigate the relationship between tree health, the severity of *C. fusipes* infection and soil characteristics, in two pedonculate oak stands. We were also able to investigate the induction and evolution of disease in two naturally infected red oak stands during a 6 years period and to relate this evolution to soil and tree characteristics.

MATERIALS AND METHODS

Study sites

Trees were sampled in four sites where *C. fusipes* showed a scattered distribution (Table 1). Two out of four sites, with *Q. robur*, were located in alluvial forests. The soil consisted in a sandy loam coarse textured layer of heterogeneous thickness covering a layer of gravel. Most roots over 1 cm in diameter did not extend into this gravel layer. In the two other sites, with *Q. rubra*, soil consisted in a layer with a coarse texture covering clay where vertical root growth was not limited (Table 1).

Table 1 . Description of the study sites.

Site (Area in France)	Oak species	No. Studied trees	No. declining oaks	Soil texture	Parental material	Soil pH
Ainvelle (Haute-Saône)	*Q. robur*	60	20	Clayey sandy silt	Gravel	4.5
Mersuay (Haute-Saône)	*Q. robur*	60	22	Sandy clayey silt	Gravel	5.2
Les Barres (Loiret)	*Q. rubra*	93	0	Sandy loam	Clay	4.0
Agre (Tarn-et-Garonne)	*Q. rubra*	51	0	Clayey silt	Clay	-

Tree sampling

Relationship between decline, *C. fusipes* infection severity and soil parameters were investigated at Ainvelle and Mersuay. Trees were selected on the basis of their crown status, rated on a 0 to 3 scale adapted from Nagelesein (1995): (0) crown healthy and opaque with dense secondary ramifications; (1) crown moderately healthy, with dead twigs present and/or gaps present in the canopy; (2) crown moderately declining, with the gaps in the canopy coalescing at the periphery of the crown and forming openings towards the outside in the upper part of the crown. The skeleton of large limbs is fully visible; (3) crown severely declining, with large dead limbs in the upper part of the crown and/or loss of more than half of fine branches. The 60 oak trees were selected to obtain dominant trees 20 being healthy oaks rated as zero or one 20 being moderately declining oaks rated as 2 and 20 being declining oaks rated as 3. The age of trees was about of 80-150 years.

Evolution and induction of the disease in a 6 years period was monitored at Les Barres and Agre. Oak trees (*Q. rubra*) were selected on the basis of their social status. Only healthy dominant and codominant oaks were selected. The age of trees varied between 40 and 70 years old at Les Barres and was of 20 years old at Agre.

Site and tree investigation

The diameter at breast height (dbh) was investigated at Les Barres in 1996 and in 2001 and at Agre in 1995 and in 2001. Oak height was measured at Agre only on 30 trees in 1995, and on every trees in 1996 at Les Barres. The number of neighbouring trees within 4 m was evaluated in 1995 only at Agre. In this stand, a silviculture trial was set up in the past and result in very heterogeneous tree density. In the 4 stands, a core of soil was excavated at the base of each tree. Soils were described especially for their variability in depth of water logging traces (traces of iron deposition or flight) and in depth of layer able to limit vertical growth of roots such as a gravel layer.

Root damage assessment

Root systems were studied for *C. fusipes* infection severity as described by Marçais et al. (1999). Briefly, the root collar and major roots were partially excavated to a depth of about 20-30 cm and a distance of about 80-100 cm from the trunk base. Lesions caused by *C. fusipes* are very characteristics and are easily detected as patches of dead bark that are orange in colour with small white fans of mycelium scattered in the necrotic tissues. On pedonculate oak , an hypertrophy of the bark is usually observed as infected bark is thickened up to 3-4 cm. In contrast, on *Q. rubra*, thickening of bark tissue is rarely observed (Marçais et al. 1999). Previous work showed that *C. fusipes* is consistently isolated from such lesions on oak roots (Guillaumin et al. 1985; Marçais et al. 1999). The infection status of each major root was assessed within the following four classes: 0) no necrosis detected; (1) superficial necrosis present, but covering less than half of the root circumference (penetration of *C. fusipes* in the bark of less than 1-2 mm); (2) necrosis covering one side of the root entirely (penetration usually 2-5 mm for *Q. robur*); (3) *C. fusipes* infection over the entire root circumference but root still alive (penetration usually more than 4-5 mm for *Q. robur*); (4) root dead with decayed wood. Diameter of each root was measured at about 10 cm from the trunk base. The root infection index of a tree was computed as: Σ (root diameter X root rating) / Σ (root diameter). This index therefore takes value from 0 to 4. Trees with an index value of 0-0.3 were rated as undamaged, those with an index value of 0.3-2 and 2-4 were rated as respectively lightly and heavily damaged trees. *C. fusipes* infection severity was rated in 2000 in Mersuay and Ainvelle, and successively in 1995 and 2001 on the same trees in Les Barres and Agre.

Statistical analysis

Root infection index and infection evolution in a 6 years period were subjected to an analysis of variance using a GLM procedure (SAS Inc. 1989). The frequency of oaks uninfected in 1995 that became infected during the 6 years period and the frequency of declining oaks were analysed by generalised linear modelling with the SAS procedure GENMOD. A binomial distribution was assumed and the logit link function was used.

RESULTS

Relationship between *C. fusipes* infection severity, pedonculate oak health and soil characteristics

In both sites, Ainvelle and Mersuay, oak decline and infection severity were significantly correlated (Table 2). The majority of oak trees rated as severely damaged by *C. fusipes* were rated as moderately or heavily declining trees (Ainvelle: 12 of 12 trees severely damaged, Mersuay: 14 of 16 trees).

Table 2. Correlation analysis between oak decline and infection severity.

Plots	Spearman correlation coefficients	Probability
Ainvelle	0.497	0.0001
Mersuay	0.428	0.0006

At Ainvelle and Mersuay, the root infection index increased significantly with the depth of water logging in the soil (Fig 1, respectively: $F = 5.14$, $df = 5$, $p < 0.001$; $F = 8.09$, $df = 4$, $p < 0.001$). Root infection index decreased when the gravel layer was close to the soil surface in Mersuay ($F = 3.23$, $df = 3$, $p = 0.029$) and when the gravel layer appeared deeper in the soil in Ainvelle ($F = 3.44$, $df = 4$, $p = 0.014$).

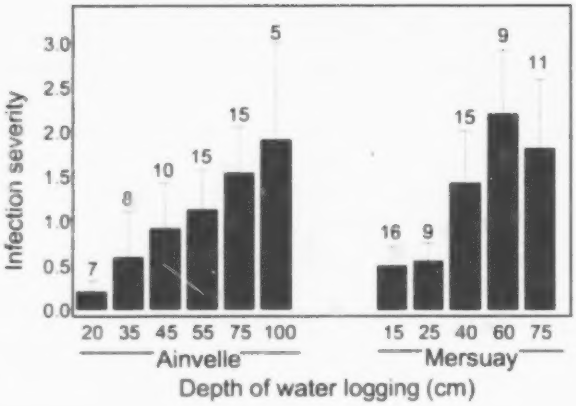

Figure 1. Mean root infection index for different depth of water logging, measured as the first traces of iron deposition, in Ainvelle and Mersuay. The bars represent the confidential interval of the mean. The number of trees in the categories is given above the bar.

In Mersuay, the percentage of declining oaks increased significantly when the water logging depth was deeper in the soil (likelihood $\chi^2 = 19.6$, df = 4, $p < 0.001$) and when the gravel layer appeared closer to the soil surface (likelihood $\chi^2 = 10.4$, df = 3, p = 0.014). However, the relation between tree crown status and depth of gravel layer was due to the fact that severely infected trees are more frequent when the gravel layer appears close to the soil surface (Fig 2). There was no significant relationship between the proportion of declining oaks and the depth of water logging or the depth of gravel layer in Ainvelle.

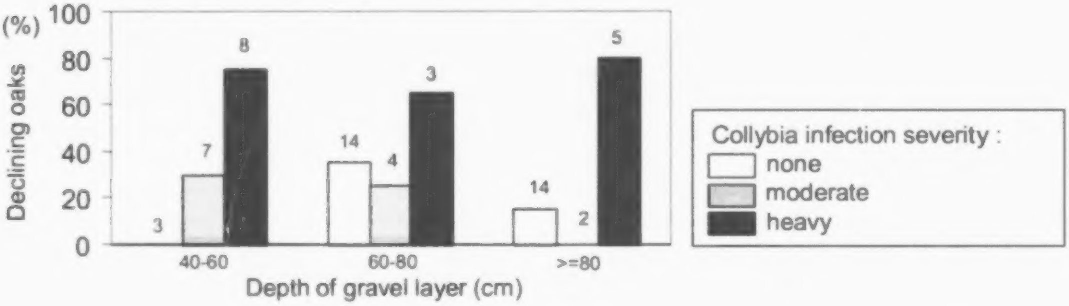

Figure 2. Proportion of declining oaks (rated as 3) in Mersuay according to their level of infection by *C. fusipes*, for different depth of gravel layer. The number of trees in the categories is given above the bars.
Effects of soil and tree characteristics on infection induction and evolution over a 6 years period, on red oaks.

Age of oaks did not appear to influence the evolution of infection at Les Barres. At Agre, in a situation very different for stand, soil and climate, disease evolution over the same period, on younger trees, was on the same range. In these two plots, evolution of infection between 1995 and 2001 did not depend on the initial level of infection in 1995. Thus, it is possible to make the hypothesis that the speed of the disease is about constant through the time. This allowed us to estimate the period required for an healthy oak (rated as 0) to become severely infected (rated as 2). This period was computed as the ratio: [(2 / infection evolution between 1995 and 2001) x 6]. The period appeared to be very long both at Les Barres and at Agre (Table 3).

Table 3. Number of years required for an uninfected red oak (rated as 0) to become severely infected (rated in 2) by *C. fusipes*.

	First quartile (25% of trees before)	Median (50% of trees in both sides)	Third quartile (25% of trees after)
Les Barres	17	35	80
Agre	17	27	70

At Les Barres, the frequency of oaks not infected in 1995 that became infected between 1995 and 2001 increased significantly with the depth of water logging (Fig. 3 ; likelihood $\chi^2 = 6.68$, df = 1, p = 0.02). This effect did not appear at Agre (Fig. 3). However, in this plot, evolution of infection on trees already infected in 1995, significantly depended on the depth of water logging (Fig. 4, F = 9.76, df = 1, p = 0.02). The infection evolution on already infected tree was not linked with water logging at Les Barres (Fig. 4).

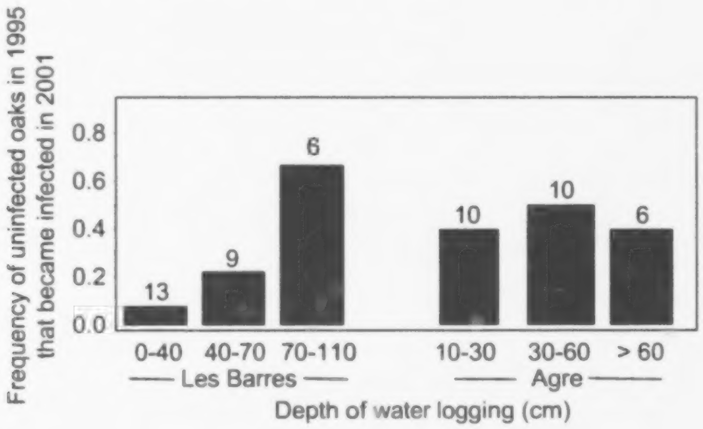

Figure 3. Frequency of uninfected oaks that became infected between 1995 and 2001 for different depth of water logging at Les Barres and Agre. The number of trees in the categories is given above the bar.

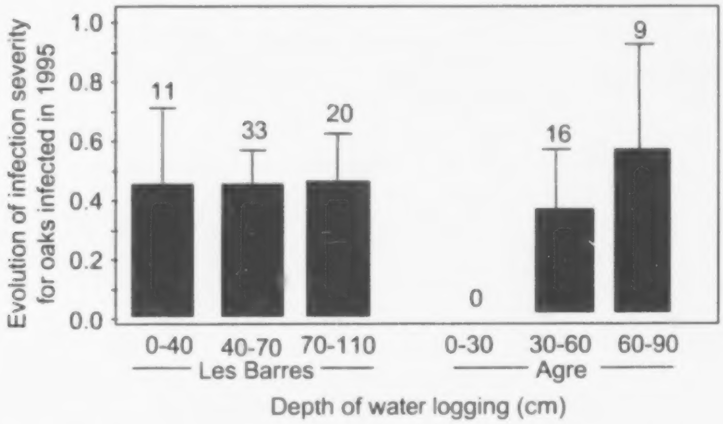

Figure 4. Evolution of root infection severity during 6 years from oaks infected in 1995, for different level of water logging at Les Barres and Agre. The bars represent the confidential interval of the mean. The number of trees in the categories is given above the bar.

At Agre, evolution of the root infection severity and frequency of uninfected oaks in 1995 that became infected in 2001 were not related to the degree of competition with neighbouring trees in 1995, evaluated either as the density of neighbouring trees within 4 m or as the ratio height/dbh. In contrast, at Les Barres, the frequency of oaks uninfected in 1995 that became infected in 2001 decreased with the degree of concurrence undergone between trees in 1995, measured as the ratio height/dbh (likelihood $\chi^2 = 5.54$, df = 1, p = 0.019).

The mean radial growth between 1995 or 1996 and 2001 decrease in the two plots when trees were moderately and severely infected by *C. fusipes* in 1995 (Table 4, Les Barres: F= 9.5, p = 0.003; Agre: F = 25.97, $p < 0.001$). No significant relationship between growth and the induction or evolution of infections could be evidenced (results not shown).

Table 4. Mean radial growth (mm / year) of red oaks between 1995 or 1996 and 2001, at Agre and Les Barres, for different degree of *C. fusipes* infection in 1995.

	Agre			Les Barres		
Infection level	Nb of trees	Mean	Std Error	Nb of trees	Mean	Std Error
None	26	5.30	0.28	28	6.95	0.31
Moderate-low	17	4.76	0.31	26	7.30	0.32
Moderate-high	8	3.54	0.33	18	6.76	0.38
Severe	0	.	.	21	4.74	0.52

DISCUSSION

Water logging is the major factor explaining distribution of *C. fusipes* infection in the four studied stands. Indeed, the parasite seems to be favoured by a reduced water logging level that might be related to oxygen availability. In vitro, the colonisation of little hazel stem segments by *C. fusipes* is strongly influenced by a good aeration of the culture. Furthermore, during an experiment of artificial inoculations, *C. fusipes* had not survived in the majority of inocula located in pots where fortuitous water logging had occurred (Marçais and Caël 2000). In a field study, Piou et al. (2001) observed that *C. fusipes* developed well on final crop trees submitted to poor water logged conditions. In the Netherlands, Nanta and Vellinga (1995) reported preferential distribution of *C. fusipes* on dry soils. Root rot fungus are often mentioned in literature as favoured by dry soil conditions. Indeed, in British Columbia, the incidence of disease induced *by Inonotus tomentosus* was higher on soils with a dry moisture regime as influenced by slope position and texture (Bernier and Lewis 1998). Whitney (1984) showed that black spruce located on dry moisture regimes are more heavily infected by *A. mellea*, than those on moist or wet sites. Kuhlman (1980) observed that root segments of loblolly pine were more decayed by *Heterobasidion annosum* when they were buried in dry soils.

The low degree of competition between trees was linked with an higher frequency of tree that became infected in the 6 years study period at Les Barres. In fact, trees subjected to a poor level of competition were located in different soil situation than those subjected to a poor competition level, with water logging appearing deep in the soil. Then, they were located in favourable conditions for *C. fusipes*.

Our study also showed that the proportion of declining pedonculate oaks increased with their severity of infection by *C. fusipes*. This observation confirms those made by Marçais et al. (2000) through other sites in France. We confirmed that in this case *C. fusipes* was the major cause of decline. Soil conditions, though they were unfavourable for *Q. robur*, appears to play a secondary role in decline.

The radial growth of red oaks of Les Barres and Agre during the 6 years period was lower on those that were the more heavily infected by *C. fusipes* at the beginning of the period. *C. fusipes* was the major factor linked with reduced growth. In those stands, Marçais and Caël (2001) have already observed, through a dendrochronological study, that trees severely infected by *C. fusipes* exhibited poor growth for many years. However, the time at which trees became infected could not be determined in this study and, as a consequence, it could not be concluded whether the root rot was the cause of the poor growth or whether oaks with poor growth had higher chance to become infected and then to develop high level of infection. Observation of the disease evolution on single trees during 6 years gives a part of the answer. Indeed, previously infected trees have a reduced growth but conversely there is no effect of tree growth on infection induction or evolution. Thus, as the parasite does not appear to be influenced by growth, that could allow us to conclude that *C. fusipes* is probably the origin of growth reduction. We were also able to estimate the period required for the parasite to induce severe damage in field conditions. Because of the slow evolution of *C. fusipes* infection, this estimation could never have been done by inoculation experiments.

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FIRE AND *ARMILLARIA*: EFFECTS ON VIABILITY AND DYNAMICS IN EASTERN OREGON, USA

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SUMMARY

This study determined the effects of prescribed burning, soil depth, antagonistic fungi (*Trichoderma harzianum* Rifai), and time since burning on the viability of *Armillaria ostoyae* (Romagnesi) Herink in wood pieces buried in the soil of a mixed-conifer forest in northeastern Oregon, USA. Red alder (*Alnus rubra* Bong) stem segments colonized with *A. ostoyae* were buried at two soil depths in plots that were burned and not burned. Half of the *Armillaria* segments were buried with segments of *T. harzianum*. Prescribed burning in the fall significantly reduced the recovery of *A. ostoyae* one day after the burn at a soil depth of 8 cm but not at a soil depth of 30 cm. Adding *T. harzianum* inoculum to the soil did not appear to reduce *A. ostoyae* recovery immediately after the fire, but *A. ostoyae* recovery appeared to decrease after several months. Differences in *A. ostoyae* recovery may also be due to the season (fall or spring) of the prescribed burns.

Keywords: *Armillaria ostoyae*, *Trichoderma*, inoculum, prescribed fire, mixed-conifers

INTRODUCTION

Fire has shaped the forests of western North America for at least 350 million years (Agee 1993). Fire return intervals have historically ranged between 40-150 years in the upper elevation, mixed-conifer forests of northeastern Oregon (Mutch et al. 1993, Fitzgerald et al. 2000). Fire exclusion and selective harvesting of pine and larch that began at the turn of the 1900s have resulted in an unprecedented abundance of *Abies grandis* (Dougl. ex D. Don) Lindl. and *Pseudotsuga menziesii* (Mirb.) Franco in many areas in the interior West (Filip 1994). Mortality from root disease and other pests is much greater in stands with more *Abies* spp. (Filip and Goheen 1984, Filip et al. 1996). As a result, these stands are overstocked with diseased, suppressed, or dead *A. grandis*, *P. menziesii*, and *Pinus ponderosa* Dougl. ex Laws. Subsequently, wildfire frequency in the western USA and even throughout the country appears to have increased with 84 690 wildfires on 43 057 ha in the USA in 2000 (National Fire News 2000).

Prescribed burning is increasingly used throughout western North America in an attempt to return fire to its natural role in the ecosystem. Only a few studies, however, have directly linked fire in the Pacific Northwest to the changes in the incidence or severity of root pathogens (Thies 1990). In the mixed-conifer forests of northeastern Oregon, *Armillaria ostoyae* is common and widely distributed (Schmitt et al. 1991). Schmitt found that *A. grandis* had the highest incidence of damage from several species of root pathogens, that *P. ponderosa* was damaged more by *A. ostoyae* than other root pathogens, and that *Pseudotsuga menziesii* was damaged by root pathogens in most sample strata and plant associations. *Armillaria ostoyae* clone sizes of 1134 ha and ages of approximately 2400 years have been reported in a study area adjacent to ours (Ferguson et al. 1999).

Biological control of *Armillaria* is difficult because effective populations of antagonistic fungi are not sustainable under normal field conditions. Filip and Yang-Erve (1997) provide a summary of the interactions between *Armillaria* spp. and *Trichoderma* spp. Because prescribed burning can influence antagonistic soil microorganisms (Margaris 1977, Parmeter 1977), controlled burns may indirectly help minimize Armillaria root disease. In central Oregon, Reaves et al. (1984) found that ash leachates from prescribed fire in ponderosa pine forests reduced the growth of *A. ostoyae* in culture. Isolates of *Trichoderma* spp. obtained from burned soils were more antagonistic to *A. ostoyae* in culture than isolates from unburned soils (Reaves et al. 1990). Hood and Sandberg (1989) showed a significant reduction in viable rhizomorph recovery for *A. limonea* (Stevenson) Boesewinkel and *A. novae-zelandiae* (Stevenson) Herink after a prescribed fire in New Zealand.

The objective of our study was to determine whether prescribed fire reduces the viability of *A. ostoyae* in wood segments artificially buried in soil in a mixed-conifer forest. We were specifically interested in four issues: (1) whether there is a significant difference in the viability of *A. ostoyae* inoculum segments between burned and unburned treatments; (2) whether the treatment difference is associated with the depth of buried segments (8 cm vs. 30 cm); (3) whether the treatment difference is associated with the presence of *Trichoderma harzianum*; and (4) whether the treatment difference is associated with time since the prescribed burning treatment (over a year).

MATERIALS AND METHODS

Research site

The study was conducted on land managed by the USDA Forest Service, Prairie City Ranger District, Malheur National Forest of Oregon (Filip and Yang-Erve 1997, Fitzgerald et al. 2000). The study site is at an elevation of 1,370 m with a west to south-facing slope. Four plant associations occur in the research area: *Abies grandis/Calamagrostis rubescens*, *A. grandis/Carex geyeri*, *A. grandis/Vaccinium membranaceum*, and *A. grandis/V. scoparium* (Johnson and Hall 1990). Stands have a dominant overstory of ponderosa pine with scattered large-diameter western larch (*Larix occidentalis* Nutt.), Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco), and grand fir. Large diameter trees are common in the study area, and stands contain dying and dead grand fir and Douglas-fir due to an outbreak of western spruce budworm (*Choristoneura occidentalis* Freeman) from 1980 to 1992. *Armillaria ostoyae*, *Armillaria* NABS X, and *Heterobasidion annosum sensu lato* occur naturally in the study area. The study area is adjacent to an *Armillaria* clone study (Ferguson et al. 2000) and a tree-density reduction study (Fitzgerald et al. 2000).

Three units in the study area were selected because of their prescribed fire treatment priority. Units A and B were selectively harvested in order to reduce excess stand density and favor ponderosa pine and western larch. Unit H was clearcut. Prescribed fire was planned in all three units in order to reduce fuel loads and create planting spots.

Because of the uneven distribution of Armillaria root disease in the study area, an isolate of *Armillaria ostoyae* from northeastern Oregon was used to artificially inoculate the treatment blocks. *Trichoderma* spp. were isolated from the soil at the study sites. A preliminary laboratory test was performed to screen for the *Trichoderma* isolate that was the most antagonistic to *A. ostoyae*; an isolate of *T. harzianum* was used as the inoculum. Red alder (*Alnus rubra* Bong.) stem segments were used as substrates for *A. ostoyae* and *Trichoderma harzianum*, and inoculum preparation has been described previously (Filip and Yang-Erve 1997)

Experimental design and plot establishment

Two blocks each were randomly established in units A, B, and H in the study area: blocks 1 and 2 in unit A, blocks 3 and 4 in unit B, and blocks 5 and 6 in unit H. Each block was designed with a split-plot configuration. The use of prescribed burning was the whole-plot factor, and soil depth and presence of *Trichoderma* were the sub-plots factors. Each 12 x 24 m sub-block (burned or unburned) contained four plots (Figure 1). Treatments were randomly assigned to the plots. Fifteen red alder stem segments colonized with *Armillaria* were buried in each plot before burning; five segments were buried for each of three sampling times (1 day, 1 month, and 2 months after burning). A *Trichoderma*-inoculum segment was buried adjacent to and touching an *Armillaria*-inoculum segment where indicated (AT). An iron stake was placed at each corner and at the center of each plot. The position of each inoculum segment was measured from the closest iron stake and recorded on a map for future sampling.

The configuration was a 2^3 factorial design; the treatments were burned or unburned, 8 or 30 cm soil depth, and presence or absence of *Trichoderma* inoculum. All segments were randomly assigned to each treatment and buried between August and September 1993. Before the prescribed fire treatment, woody debris was evenly scattered over each plot so that burning was uniform.

Prescribed burning

Unfortunately, local weather and the availability of personnel at the Prairie City Ranger District affected our ability to conduct prescribed burning. Instead of burning all six blocks in the fall of 1993 as planned, the fire treatment occurred at three different times over two years. Blocks 5 and 6 in H unit were burned for an hour on 27 October 1993. The woody debris and litter layer on both blocks were barely consumed; soils were moist to the touch. On 31 October 1993, the burned plot of block 6 was reburned for four hours because of the poor original burn; the first sampling of segments occurred the next day, the second sampling occurred in June 1994, and the third in August 1994.

Block 3 in unit B and block 5 in unit H were burned on 8 June 1994, nine months after inoculation. The fire lasted one hour, was extinguished with water, and completely consumed the woody debris and litter layer. We began sampling the next day, sampled a second time in July, and a third time in August.

Blocks 1, 2, and 4 in units A and B were burned on 27 October 1994, one year after inoculation. The fire lasted one hour and completely consumed the woody debris and litter layer. Water was used on all plots to extinguish the fire. Sampling for Blocks 1, 2, and 4 was done only on the day after the burn. These three blocks received the same treatment, so data could be statistically analyzed.

Inoculum recovery

Following each prescribed fire treatment, five segments from each treatment were randomly selected in order to test the viability of *A. ostoyae* at each sampling time. Segments were excavated individually, bagged in separate paper bags for each treatment, and stored at 1-2°C until they were processed in the laboratory. We attempted to isolate the fungi by splitting each segment aseptically and plating five wood chips (1 x 1 x 1 mm) onto a selective medium for *Armillaria* (2% malt agar with 1.5 ppm each of prochloraz, benomyl, and thiabendazole; 30 ppm rose bengal; and 100 ppm streptomycin sulfate; Goldfarb et al. 1989). The recovery rate of *A. ostoyae* was scored from 0-5 based on the number of isolates recovered from that segment. The number of *A. ostoyae* isolates recovered was converted to 0-100% scale. Treatment means were the average of the 15 segments in that treatment.

Statistical analysis

Percent recovery of *A. ostoyae* was tabulated by burning treatment, soil depth, and presence of *Trichoderma*. Data from blocks 1, 2, and 4 were examined by using analysis of variance for a 2^3 factorial with an F test ($P<0.05$; SAS Institute, Inc. 1994). We explored the main and interactive effects of prescribed burning, depth of inoculum, and the presence of *Trichoderma* on the percentage recovery of *A. ostoyae*. Comparisons were made between all combinations of treatments.

RESULTS

There were no significant differences among blocks 1, 2, and 4 ($P = 0.94$) in the percentage recovery of *Armillaria* immediately after the prescribed burn in October 1994. The effect of prescribed burning on *Armillaria* viability was significant ($P = 0.0138$; Table 1). The interaction between the prescribed burn and soil depth on *Armillaria* viability was also significant ($P = 0.0138$). The 3-way interaction of prescribed burning, soil depth, and the presence of *Trichoderma* ($P = 0.3003$), the interaction between prescribed burning and the presence of

Trichoderma (P = 0.4313), and the interaction between soil depth and the presence of *Trichoderma* (P = 0.9193) did not significantly affect the viability of *Armillaria*.

Table 1. Results of an analysis of variance of the effects of prescribed burning (Burn), soil depth, and presence of *Trichoderma* (*Trich*) on the viability of *A. ostoyae* inoculum one day after fall treatment.

Source	NDF[1]	DDF[1]	Type III F[1]	Pr > F[1]
Burn	1	14	7.92	**0.0138**
Soil depth	1	14	0.06	0.8059
Trich	1	14	0.14	0.7181
Burn x Depth	1	14	7.92	**0.0138**
Burn x *Trich*	1	14	0.66	0.4313
Dept x *Trich*	1	14	0.01	0.9193
Burn x Depth x *Trich*	14	1.16	0.3003	

[1] NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; Type III F = Type III F-value; and Pr> F = probability greater than F-value. Bolded values are significant.

Prescribed burning and soil depth

The recovery of *Armillaria* decreased significantly from 36 to 5% at soil depths of 8 cm in burned plots but remained unchanged at soil depths of 30 cm (Table 2). The recovery of *Armillaria* was lowest in the burned and highest in the unburned treatments at a soil depth of 8 cm. The mean recovery of *Armillaria* at the 8 cm depth with prescribed burning was 5% which is not statistically different than 0% recovery (P = 0.43); thus *Armillaria* at a soil depth of 8 cm had virtually no viability after the prescribed burning. The mean recovery of *Armillaria* in unburned plots was 36% at 8 cm and 19% at 30 cm; however, depth itself did not have a significant effect on the percentage recovery of *Armillaria* in unburned plots (P = 0.8059; Table 1).

Table 2. The percentage recovery of *Armillaria ostoyae*, at two soil depths and at burned and unburned sites one day after fall treatment in northeastern Oregon.

Treatment	Soil depth (cm)	Mean[1]	SE
Burned	8	5 (0.23)a[2]	0.29
Burned	30	19 (0.95)ab	0.29
Unburned	8	36 (1.83)b	0.29
Unburned	30	19 (0.95)ab	0.29

[1] Means are the percentage and actual number (in parentheses) of viable isolations of *A. ostoyae* out of a total of five attempts from each of 15 segments per treatment.

[2] Means followed by a different letter are significantly (P<0.05) different according to ANOVA (SAS Institute Inc. 1994).

Season of burning

Block 6 in Unit H was burned in the fall 1993 and sampled three times: fall 1993, spring 1994, and fall 1994. Because this was the only block effectively treated in fall 1993, there was no statistical analysis. In plots treated with prescribed burning, the recovery of *Armillaria* appeared to remain the same at the 30 cm soil depth regardless of the time of sampling; however, recovery decreased at the 8 cm depth at the second sampling. In unburned plots, the recovery of *Armillaria* at a depth of 8 cm was higher than at 30 cm at the second and third samplings.

Following the burning treatment, the recovery of *Armillaria* appeared to decrease with time in the presence of *Trichoderma* but increased in the absence of *Trichoderma*. Without the burning treatment, recovery of *Armillaria* increased at the second sampling, then decreased at the third sampling, especially in the presence of *Trichoderma*.

Block 3 in Unit B was burned in spring 1994 and sampled three times: June, July, and August 1994. Block 5 in Unit H was also burned in the spring, but the unburned plot was accidentally destroyed. The spring burn seemed to have no effect on the recovery of *Armillaria* during the sampling period. At the second and third sampling, *Armillaria* recovery in burned plots at the 8 cm depth was higher than recovery at 30 cm. The presence or absence of *Trichoderma* appeared to have little affect on *Armillaria* recovery.

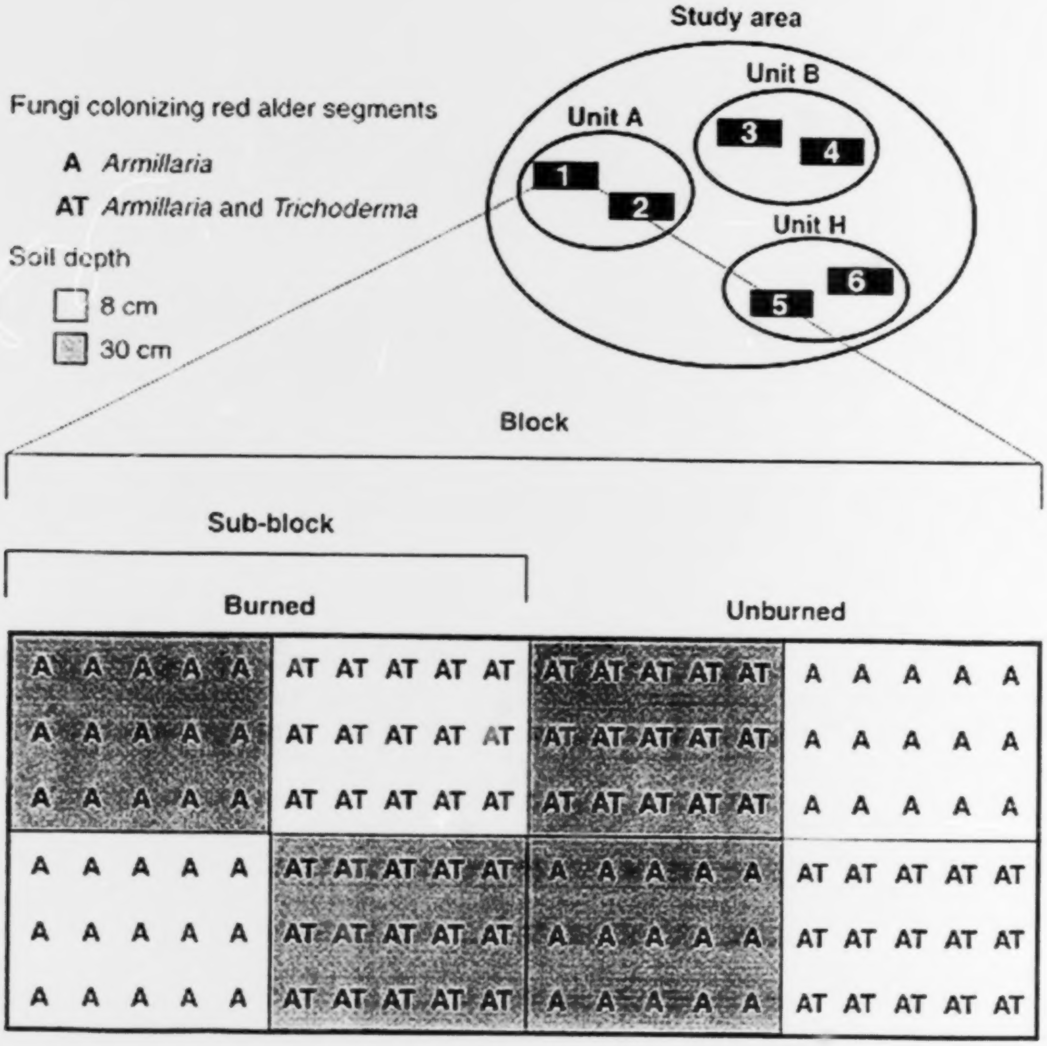
DISCUSSION AND CONCLUSION

There was a significant reduction in *Armillaria* recovery immediately following a fall burn. The recovery of *Armillaria* was lowest (5%) in burned plots and highest (36%) in unburned plots in segments at a soil depth of 8 cm. The results of this field study suggest that the heat from the fire was able to kill a high proportion of *Armillaria* at a soil depth of 8 cm but not at 30 cm. The fact that most segments were scorched by the fire in all three blocks further supported the conclusion that the reduction in *Armillaria* recovery was caused by heat. As mentioned previously, Hood and Sandberg (1989) reported that there was a highly significant reduction in the yield from isolates of rhizomorphs, although no significant change in rhizomorph frequency, after felling and burning of forest vegetation in New Zealand.

Trichoderma harzianum can kill *Armillaria* mycelium in vitro. The shift in the microbial balance from ash leachates in forest soils (Reaves et al. 1990) that favors the antagonist *Trichoderma* following a prescribed fire is a rather slow process compared to lab tests. We did not find a decrease in *Armillaria* with *Trichoderma* in samples taken immediately after the fire, although 8-10 months after the prescribed burning we found a decrease in *Armillaria* recovery. Additional research is needed in order to evaluate whether the reduction in *Armillaria* recovery is short-term. Also, there are large natural populations of *Trichoderma* spp. in forest soils that we did not monitor.

There was no apparent difference in the recovery of *Armillaria* between burned and unburned plots after the spring burning treatment. This differs from the results after the fall burning; we found a significant difference between burned and unburned plots at the 8 cm soil depth. More studies are needed to establish the timing and intensity of fire that are necessary to effectively reduce *Armillaria* recovery.

Woody roots that are naturally infected with *Armillaria* are normally present at depths much greater than were inoculum segments in our study. Obviously, an operational burn with conditions similar to our study would only destroy a small portion of the total *Armillaria* inoculum. Unless the burn was hotter or more penetrating, a situation that could lead to soil damage and significant nutrient loss, the direct effects of prescribed fire on *Armillaria* populations appear to be negligible. The long-term effect of burning as related to changes in *Trichoderma* populations and subsequent indirect effects on *Armillaria*, however, may be more important. Long-term studies that explore the effects of prescribed burning on naturally infected roots in soil need to be conducted. Advanced *A. ostoyae* clone sizes and ages in northeastern Oregon (Ferguson et al. 1999) testify to the population stability and resilience through centuries of fire and forest structural changes.


Study area
Unit A
Unit B
Unit H
1
2
3
4
5
6
Fungi colonizing red alder segments
A Armillaria
AT Armillaria and Trichoderma
Soil depth
8 cm
30 cm
Block
Sub-block
Burned
Unburned

A A A A A	AT AT AT AT AT	AT AT AT AT AT	A A A A A
A A A A A	AT AT AT AT AT	AT AT AT AT AT	A A A A A
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Figure 1. Diagram of the plot configurations and randomized treatment applications within each block. Red alder segments, colonized by *Armillaria* (A) were buried 8 or 30 cm under the soil surface (soil depth). In some cases, a segment colonized by *Armillaria* and a segment colonized by *Trichoderma* were buried together (AT). The plots in which these segments were buried were subsequently subjected to prescribed burning treatments.

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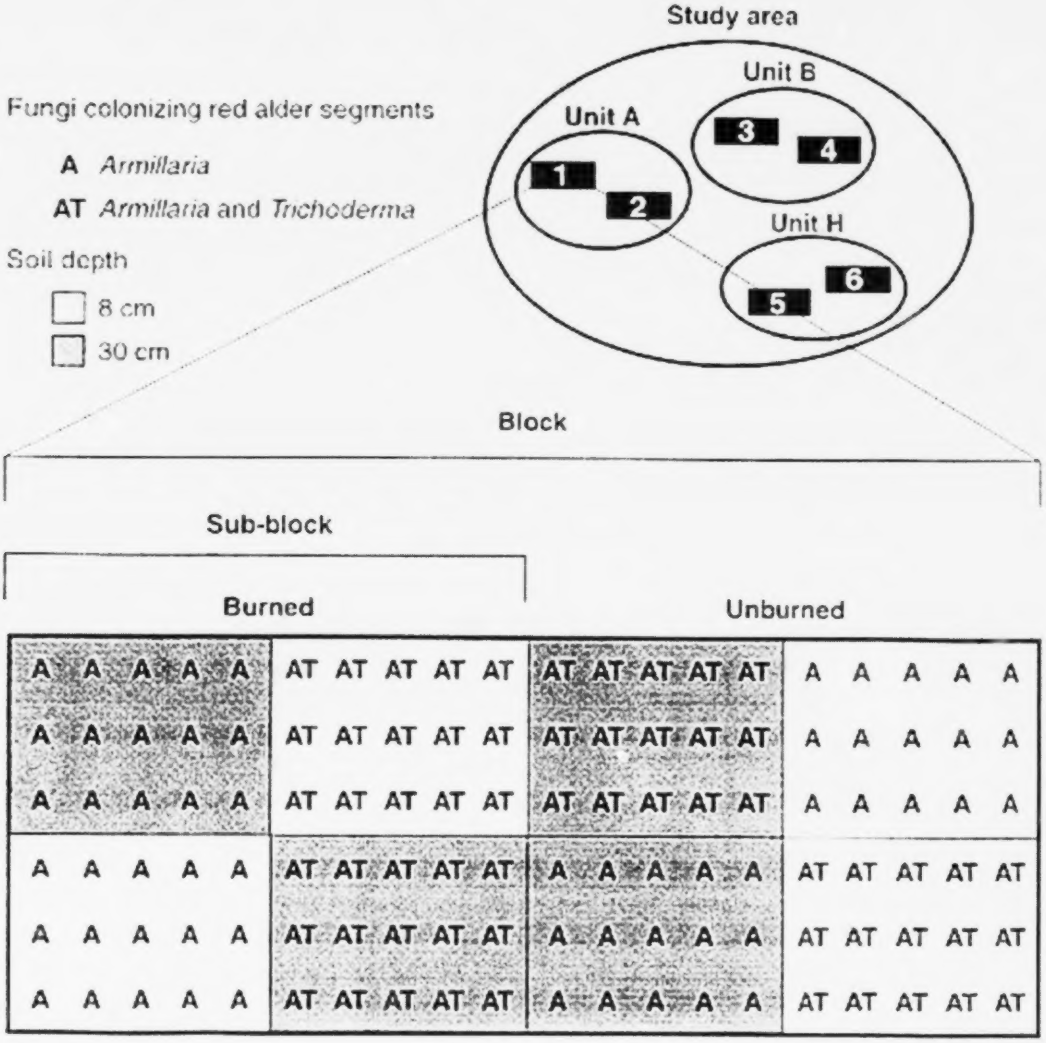
Study area
Unit B
Unit A
Unit H
1
2
3
4
5
6
Fungi colonizing red alder segments
A *Armillaria*
AT *Armillaria* and *Trichoderma*
Soil depth
☐ 8 cm
▨ 30 cm
Block
Sub-block

Burned		Unburned	
A A A A A	AT AT AT AT AT	AT AT AT AT AT	A A A A A
A A A A A	AT AT AT AT AT	AT AT AT AT AT	A A A A A
A A A A A	AT AT AT AT AT	AT AT AT AT AT	A A A A A
A A A A A	AT AT AT AT AT	A A A A A	AT AT AT AT AT
A A A A A	AT AT AT AT AT	A A A A A	AT AT AT AT AT
A A A A A	AT AT AT AT AT	A A A A A	AT AT AT AT AT

Figure 1. Diagram of the plot configurations and randomized treatment applications within each block. Red alder segments, colonized by *Armillaria* (A) were buried 8 or 30 cm under the soil surface (soil depth). In some cases, a segment colonized by *Armillaria* and a segment colonized by *Trichoderma* were buried together (AT). The plots in which these segments were buried were subsequently subjected to prescribed burning treatments.

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EFFECTS OF NUTRIENTS ON *ARMILLARIA* ROOT DISEASE IN GREENHOUSE-GROWN LODGEPOLE PINE (*PINUS CONTORTA*)

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SUMMARY

Armillaria root disease is thought to occur in nutrient stressed trees; however, some studies have found a greater incidence of Armillaria root disease on high versus low productivity sites and in fertilized versus non-fertilized plantations. A greenhouse experiment was initiated to test whether young lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) grown under low nutrient conditions were more susceptible to Armillaria root disease than trees grown under optimal nutrient conditions. Lodgepole pine trees, grown in the greenhouse and fertilized with either full- or quarter-strength Hoagland's solution were inoculated with *Armillaria ostoyae* (Romagn.) Herink. Trees from each treatment were examined 3, 6, 9, and 12 months after inoculation for evidence of infection. Disease incidence was greater in trees grown with the full-fertilizer treatment than those grown with the quarter-strength fertilizer treatment; however, inoculum survival was better in the quarter treatment. Infection was not related to size of the tree. The increased incidence of *Armillaria* root disease in the full-strength fertilizer treatment may be related to changes in the chemical constituents of the tree root or the fungus's ability to use the food base in creating rhizomorphs or mycelia.

Keywords: *Armillaria* root disease, *Armillaria ostoyae*, stress, nutrient

INTRODUCTION

Armillaria root disease is one of the most important diseases of forest trees and occurs in all forested regions of the globe on a variety of host species (Wargo and Shaw 1985). In Canada, *Armillaria* root disease is found in all forest regions and can cause significant losses in coniferous and deciduous tree stands of all ages (Hiratsuka 1987, Myren 1994).

Until recently, *Armillaria* root disease was considered to be a disease of severely stressed trees (Wargo and Harrington 1991) and the incitant, *Armillaria mellea* (sensu lato), was thought to be a secondary pathogen. With the discovery that there are many different species of *Armillaria,* it became apparent that some species such as *Armillaria ostoyae* (Romagn.) Herink were primary pathogens (Gregory et al. 1991). *Armillaria ostoyae* is the principal species of *Armillaria* found in conifers in western North America (Wargo and Shaw 1985, Mallett 1992). Little is known about the environmental conditions that must be present for these primary pathogenic species of *Armillaria* to damage trees. A higher incidence of *Armillaria* root disease is thought to occur in trees growing on nutrient deficient sites (Wargo and Harrington 1991). The disease in conifers has been associated with low soil nitrogen (Singh 1983, Entry et al. 1986, 1991). Very few studies have examined the effect of edaphic factors on *A. ostoyae* and *Armillaria* root disease (Shields and Hobbs 1970, Entry et al. 1986, 1991, Blenis et al. 1989). In a greenhouse study, soil type significantly affected the inoculum viability, rhizomorph production, and amount of *Armillaria* root disease in lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) caused by *A. ostoyae* (Blenis et al. 1989). Seedlings grown in a soil from a highly productive site (site productivity was measured by periodic annual increment) had the least incidence of disease whereas those grown in soil from a low productivity site had the greatest incidence of disease. In contrast, a life table study of lodgepole pine in west central Alberta showed that there was a greater incidence of mortality due to *Armillaria* root disease on high versus low productivity sites (Ives and Rentz 1993).

In this study we report on the effect of soil nutrients on *Armillaria* root disease caused by *A. ostoyae*, in greenhouse-grown lodgepole pine seedlings. Specifically we tested whether lodgepole pine seedling grown under low nutrient conditions were more susceptible to *Armillaria* root disease than seedlings grown with optimal nutrients. Seedling heights, diameters, shoot and root dry weights as well as elemental analysis of seedling tissues were measured to determine the response to nutrient stress.

MATERIALS AND METHODS

Isolate identification

Two isolates of *Armillaria ostoyae* (NoF 1076 and NOF 898) isolated from basidiocarps were used in the experiments. *Armillaria* species identifications were made by haploid pairing tests using basidiospore isolates from basidiocarps (Anderson and Ullrich 1979). Diploid isolates used were isolated from the stipe tissue of basidiocarps. All isolates were grown on carrot agar (Mallett and Colotelo 1984).

Inoculum preparation

Inoculum was prepared from branch segments of *Populus tremuloides* Michx. (approximately 10 × 2 cm). Branch segments were autoclaved in metal surgical instrument trays (46 × 12.5 × 6 cm) containing 300 ml of water (60 branch segments per tray) for 60 min. Three hundred milliliters of malt extract-dextrose-peptone broth (MPDB) (3% w:v malt extract 2% dextrose, 0.5% peptone, in distilled water) was then added to each tray before autoclaving for an additional 20 min. Isolate cultures grown on carrot agar were comminuted in 100 ml of sterile distilled water in a sterile blender and then added aseptically to the trays containing the branch segments and MDPB. Inoculated branch segments were kept at 25°C in the dark for 3 months.

Fertilizer experiment

Lodgepole pine seeds, collected near Hinton, Alberta, were seeded in 3 litre plastic pots containing limed peat moss (pH 5), three seeds per pot. The exposed surface of the peat was covered with horticultural grit (approximately 0.5 cm in depth) to control bryophyte growth. After the seedlings had emerged, seedlings were thinned to one seedling per pot. Then an inverted 2 × 25 cm test tube was inserted into the soil parallel to each seedling's stem. Seedlings were grown in a greenhouse compartment with artificial light (high pressure sodium vapour lamps, 400 W, with an intensity of 363 $\mu mol\ m^{-2}s^{-1}$) with a photoperiod of 18 h and day and night temperatures of 25°C and 20°C, respectively. The seedlings were watered twice weekly and fertilized once a week with full-strength Hoagland's No. 2 solution (1 ml of 1 M KH_2PO_4, 5 ml of 1 M KNO_3, 5 ml of 1 M $Ca(NO_3)_2$ 2 ml of 1 M $MgSO_4$, 0.5% Fe tartrate 1 ml microelement stock [0.286% H_3BO_3, 0.181% $MnCl_2{\cdot}4H_20$, 0.008% $ZnSO_4{\cdot}7H_20$, $H_2MoO_4{\cdot}H_20$] 1 L distilled water) (Hoagland and Arnon 1950) for 4 months.

Four months after seeding, half of the 384 seedlings were fertilized with full-strength Hoagland's and the remaining seedlings were fertilized with quarter-strength Hoagland's solution. Three hundred milliliters of fertilizer solution were dispensed to each pot. Seedlings in each fertilizer treatment were further subdivided into those that received isolate NOF-1076, NOF-898, or a sterile inoculum piece. The seedlings were inoculated when they were 6-months old by removing the test tubes and replacing them with inoculum pieces. The seedlings in the experiment were assigned to a treatment in one of four blocks (greenhouse benches) in a randomized complete block design. The heights and diameters (measured at the soil line) of the seedlings were recorded at the time of inoculation.

Sixteen seedlings from each treatment were measured and examined 3, 6, 9, and 12 months after inoculation. Heights and diameters of the seedlings were recorded before the roots were examined for symptoms and signs of *Armillaria* root disease. Those seedlings with resinous lesions and an attached rhizomorph or a characteristic mycelial fan of *Armillaria* were considered diseased. Seedlings that died during the experiment were examined for fans of mycelium beneath the bark. Cultures were made from mycelial fans to confirm that it was *A. ostoyae*. Species identification was made using the method of (Hopkin et al. 1989). Root and shoot dry weights were obtained by separating roots, stems, and foliage and then drying them in ovens at 60°C to a constant weight. Inoculum pieces were retrieved and examined. If rhizomorphs, yellow stringy rot, and/or a mycelial fan were observed, the inoculum was considered viable.

Elemental analysis

Total nitrogen (N), phosphorus (P), sulfur (S), calcium (Ca), magnesium (Mg), and potassium (K) were determined for the needles, stems, and roots of all seedlings. Total nutrient analyses were performed on oven-dried samples that had been ground in a Wiley mill and passed through a 0.25 mm sieve. The samples were digested with HNO_3-H_2O_2-HCl in a microwave oven (Kalra et al. 1989) and analysed for S, P, Ca, Mg, and K. Total N was determined using a modified Kjeldahl digestion technique with a Tecator Kjeltec 1030 automated system (Kalra and Maynard 1991). Samples were digested in an aluminum block digester using an H_2SO_4 and K_2SO_4-$CuSO_4$ catalyst mixture (Kjeltab).

Statistical analysis

Armillaria root disease incidence and inoculum survival data were analysed using a linear model in the Categorical data modeling procedure (CATMOD) of the Statistical Analysis System (SAS) Institute, Inc. (Statistical Analysis System Institute, Inc. 1990. SAS/STAT user's guide. Vol 1,2. Ver. 6. 4th ed. SAS Institute, Cary, North Carolina). The Chi-square statistic was used to analyze the disease incidence and inoculum survival data because it does not assume any underlying distribution of the data. The foliar, stem, and root nutrient and the growth data were analyzed by analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS. Least square means (LSMEANS), using Fisher's protected least significant difference, was utilized for mean comparisons where significant effects were detected by the ANOVA.

RESULTS

Different concentrations of Hoagland's solution had an effect on *Armillaria* root disease in lodgepole pine. There was a greater incidence of disease in the full-strength fertilizer treatment compared to the quarter-strength treatment (Table 1, Chi-square = 4.03, $P = 0.04$). Only seedlings that were exposed to viable inoculum were considered in the analysis. A greater incidence of disease occurred in the seedlings that received the full-strength fertilizer treatment at each sampling date. There was no difference in the incidence of Armillaria root disease (infected + dead seedlings) caused by the two isolates (Chi-square = 1.97, $P = 0.16$); however, isolate NOF-1076 caused mortality, NOF-898 did not. There was no significant difference in the amount of mortality between the full- and quarter-strength treatments (7.3 and 6.7%, respectively) or when it occurred.

Inoculum in the quarter-strength treatment had slightly greater survival than the inoculum in the full-strength treatment except at the second sampling date (Table 2, Chi-square = 3.62, $P = 0.06$). There was a significant difference in inoculum survival between isolates regardless of treatment; inoculum pieces of NOF-898 did not as survive as well as NOF-1076 (Chi-square = 23.3, $P = 0.00$).

Selected growth characteristics of non-inoculated seedlings in each of the treatments after one year were compared (Fig.1). There were no differences in dry root weights, height and diameters (at the beginning of the experiment) between the two treatments; however, there were differences in the dry shoot weights and the heights and diameters of the seedlings after 12 months growth. Seedlings in the full-strength treatment were larger than seedlings in the quarter-strength treatment. There was no difference between infected and non-inoculated seedlings in dry root weight ($P = 0.14$), dry shoot weight ($P = 0.71$), height ($P = 0.61$) or diameter ($P = 0.97$).

The concentrations of N, P, and S in the needles, stems and roots of non-inoculated seedlings fertilized with the full-strength Hoagland's solution were higher than in the needles, stems and roots of seedlings fertilized with quarter-strength Hoagland's solution (Table 3). There were no differences in the concentrations of Ca, Mg, and K in any plant part between nutrient treatments (data not shown).

DISCUSSION

Armillaria root disease was greatest and occurred earlier in the full-strength treatment compared to the quarter-strength treatment. Seedlings in the quarter strength treatment were nutrient stressed as shown by the significant difference in seedling height, diameter, and nutrient (N, P, S) content of the seedlings as compared to the full strength seedlings. Increased *Armillaria* root disease has been observed in forest stands fertilized with N (Rykowski 1981a, b, Entry et al. 1991b). Rykowski (1981a, b) found that the mortality of seedlings was higher in two of three Scots pine plantations fertilized with N, P, K, Mg and Ca. Entry et al (1991b) observed the highest rate of infection by *A. ostoyae* in a Douglas-fir stand thinned and fertilized with 360 kg N ha^{-1} compared to unthinned, unfertilized and thinned, unfertilized stands. In addition, a lifetable study of young lodgepole pine in west-central Alberta found a greater incidence of *Armillaria* root disease on high productivity sites versus medium and low productivity sites (Ives and Rentz 1993).

In contrast, severe nitrogen deficiency has been associated with a higher incidence of *Armillaria* root disease (Entry et al. 1986, 1991a, Singh 1983). Under the severe N deficient conditions created in these studies, seedlings may be more susceptible to *Armillaria* root disease. It is unlikely, however, such severe conditions would exist in most forests. We provided sufficient N to maintain the seedlings, although growth in the quarter-strength treatment was limited and the N concentration of the needles was in the range considered deficient (Morrison 1974, Maynard and Fairbarns 1994). The contrasting results could also be due to other factors, such as virulence of the *A. ostoyae* isolates involved, in addition to nutrient availability and the severity of the nutrient stress.

Added N may increase *Armillaria* root disease by either effecting the chemical constituents of the tree root as suggested by Entry et al. (1991a, b) or effecting the fungus directly. Entry et al (1991a, b) suggested nutrient imbalances (either too little or too much) may have decreased the concentration of phenolic and lignin compounds in root bark tissue while increasing the sugar content making the tree more susceptible to *A. ostoyae*. Conversely, the effect of the increased nutrients in the full-strength treatment may have been on the fungus's ability to use the food base in creating rhizomorphs or mycelia (in the case of root to inoculum piece contact) with greater inoculum potential.

There was a trend for lower inoculum survival in the full-strength treatment than in the quarter-strength treatment. The decrease in inoculum survival in the full-strength treatment may have been associated with an increase in the populations of other soil microorganisms due to a higher concentration of soil nutrients (Alexander 1977, Cook and Baker 1983). These organisms may have been antagonistic to *A. ostoyae*.

Edaphic factors such as nutrients may not only predispose seedlings to *Armillaria* root disease but may affect inoculum quality. Abundant soil nutrients may increase the ability of *A. ostoyae* to utilize its food base quickly, possibly by decreasing the carbon to nitrogen ratio, creating a greater inoculum potential. Therefore, seedlings growing on high productivity sites, with respect to nutrients, may be at greater risk of contracting *Armillaria* root disease than seedlings growing on less productive sites. This will require further investigation, however, before appropriate management prescriptions can be made for specific sites.

Table 1. The effect of full- and quarter-strength Hoagland's solution on *Armillaria* root disease in lodgepole pine seedlings caused by two isolates of *Armillaria ostoyae* (NOF-1076 and NOF-898) three to twelve months after inoculation.

Isolate	Fertilizer treatment	Health Status	Months after inoculation				Total	Percentage (%)
			3	6	9	12		
NOF-1076	Full strength	infected	1	0	4	7	12	21.8
		dead	0	0	3	1	4	7.3
		healthy	12	14	9	4	39	70.9
		Total	13	14	16	12	55	100.0
NOF-1076	Quarter strength	infected	0	1	4	2	7	11.7
		dead	0	0	2	2	4	6.6
		healthy	15	13	9	12	49	81.7
		Total	15	14	15	16	60	100.0
NOF-898	Full strength	infected	0	5	0	3	8	22.2
		dead	0	0	0	0	0	0.0
		healthy	9	5	8	6	28	77.8
		Total	9	10	8	9	36	100.0
NOF-898	Quarter strength	infected	0	1	0	3	4	9.5
		dead	0	0	0	0	0	0.0
		healthy	10	6	13	9	38	90.5
		Total	10	7	13	12	42	100.0

* Count Data

Table 2. The effect of full- and quarter-strength Hoagland's solution on inoculum survival of two isolates of *Armillaria ostoyae* (NOF-1076 and NOF-898).

Isolate	Fertilizer treatment	Status	Months after inoculation				Total	Percentage (%)
			3	6	9	12		
NOF-1076	Full strength	inviable	3	2	0	4	9	14.1
		viable	13	14	16	12	55	85.9
		Total	16	16	16	16	64	100.0
NOF-1076	Quarter strength	inviable	1	2	1	0	4	6.2
		viable	15	14	15	16	60	93.8
		Total	16	16	16	16	64	100.0
NOF-898	Full strength	inviable	7	6	8	7	28	43.7
		viable	9	10	8	9	36	56.3
		Total	16	16	16	16	64	100.0
NOF-898	Quarter strength	inviable	4	9	3	4	20	32.3
		viable	10	7	13	12	42	67.7
		Total	14	16	16	16	62	100.0

* Count Data

Table 3. Total Nitrogen (N), Phosphorus (P), and Sulfur (S) concentrations (g kg^{-1}) in needles, stems, and roots of 16-month-old lodgepole pine seedlings treated with quarter- and full-strength Hoagland's solution. Values are means ± standard error.

Nutrient	Needles		Stems		Roots	
	Full strength	Quarter strength	Full strength	Quarter strength	Full strength	Quarter strength
N	13.58 ± 0.43	7.38 ± 0.40	12.28 ± 0.42	5.72 ± 0.39	13.38 ± 0.50	5.13 ± 0.47
	P=0.0001		*P*=0.0001		*P*=0.0001	
P	1.53 ± 0.04	0.87 ± 0.04	1.98 ± 0.11	1.23 ± 0.10	1.95 ± 0.09	1.13 ± 0.08
	P=0.0001		*P*=0.0001		*P*=0.0001	
S	2.07 ± 0.12	1.51 ± 0.11	1.70 ± 0.08	1.25 ±0.08	2.48 ± 0.11	1.47 ± 0.10
	P=0.001		*P*=0.0002		*P*=0.0001	

* *P* value for the comparison between full- and quarter-strength treatments

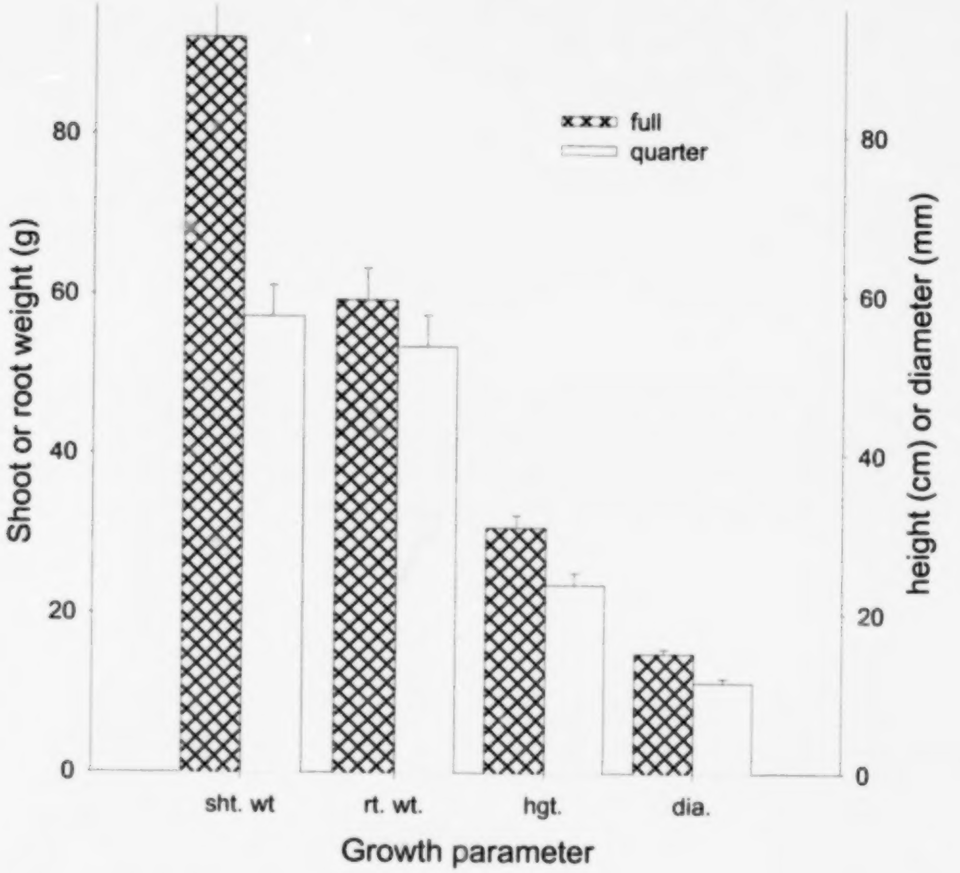

Figure 1. Means of growth parameters of non-inoculated greenhouse-grown lodgepole pine seedlings treated with full- and quarter-strength Hoagland's solutions (sht. wt.= shoot weight, rt. wt. = root weight, hgt. = height, dia. = diameter). Different letters for each growth parameter indicate a statistically significant difference (P = 0.05). Vertical bars = ± 1 S.E.

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INVESTIGATIONS ON THE DISTRIBUTION AND ECOLOGY OF *ARMILLARIA* SPECIES IN ALBANIA

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ABSTRACT

Five *Armillaria* species were identified in a nation-wide survey in Albania:

Armillaria mellea sensu stricto was found on several conifers and broad-leaved trees in most of the areas examined, except the high altitudes (above 1100-1200 m) in northern Albania. It was found to cause some damage to fir, oak, beech, chestnut, poplar, and hop hornbeam, and significant damage to fruit trees and grapevine.

Armillaria gallica was common in conifer and broad-leafed-tree forests at altitudes from 600 m to 1600 m, less common at lower altitudes on oaks. The fungus is a weak parasite or a saprophyte of forest trees and was only occasionally found on cultivated plants.

Armillaria ostoyae was rare in central and southern Albania, but is widely distributed in the forests of the northern Albania, causing significant damage to several conifers at altitudes from 600 to 1800 m's', occasionally also at lover altitudes. It was not found in fruit orchards and vineyards.

Armillaria cepistipes was recorded at high altitudes from 800 to 1800 m's', mostly as a saprophyte in conifer and broad-leafed-tree forests, predominating in beech and Silver fir forests.

Armillaria tabescens was found mostly in oak forests at altitudes from 0 to 900 m. In fruit orchards, it was occasionally found to cause disease on almond trees and pear trees (*Prunus* spp.).

Keywords: Agaricales, *Armillaria mellea, A. gallica, A. tabescens A. ostoyae, A. cepistipes*, compatibility test, distribution, ecology, host preference

INTRODUCTION

Seven *Armillaria* species have been described in Europe. Six of them are wood-decay fungi with a wide distribution and they are of great ecological and economical importance (Kile et al. 1991; Guillaumin et al. 1993). The distribution and ecological characteristics of *Armillaria* species are fairly well known throughout western and northern Europe, but limited information is available from eastern Europe and from the Balkan region, including Albania.

Six *Armillaria* species have been reported from Slovenia, the northernmost Balkan country (Munda 1997), and five species have been found in the southernmost country, Greece (Tsopelas 1999). The rare European species, *A. ectypa* (Fr.) Lamoure was not found in either of these countries, and *A. borealis* Marxm. & Korhonen was also absent in the material investigated from Greece.

Five *Armillaria* species have been reported from Albania: *A. mellea* sensu stricto (Vahl: Fr.) Kummer, *A. gallica* Marxm. & Romagn., *A. tabescens* (Scop.) Emel, *A. ostoyae* (Romagn.) Herink and *A. cepistipes* Velen. Some aspects of their distribution and ecology have been published in preliminary reports in Albanian language (Lushaj 1992; Lushaj and Shyti 1995; Intini and Lushaj 1998). The work reported here gives more detailed information on the distribution, ecology and host range of *Armillaria* species in Albania. Furthermore, it focuses on the damage caused by these fungi in different types of forests, fruit orchards and vineyards.

MATERIALS AND METHODS

Study sites and hosts

The presence of *Armillaria* species was investigated from 1990 to 2000, both in forest and agricultural areas. Permanent experimental plots were established in many parts of Albania in different types of forests, fruit orchards and vineyards at altitudes from sea level to 2100 m, at different levels of surveys. In addition, surveys were made in all main districts of the country, from the Tropoje and Shkoder districts in the north to Kosove borders in the east and to the Gjirokaster and Korçe districts in the south, to Greece borders.

The surveyed forests, orchards and vineyards were selected and permanent sample plots were established, for the first period, during the years 1992-1995, in a non-systematic manner in some areas, in the most attacked trees (Lushaj 1992; Lushaj et al. 1995), and in a systematic manner on permanent sample monitoring plots by a network gird 10 x 10 km of the National Assessment and Monitoring System in Albania, for the second and third periods, during the years 1996-2000. The investigations concerned largely natural forests, while a limited number of reforested areas were examined. Efforts were made to include many different forest types from several locations. Altogether, 42 different forest areas were investigated, occurring in 40 mountainous forest areas (data available from the author) and on two non-mountainous forest areas. The monitoring plots (50 x 50 m = 2500 m^2) were laid out on a grid 10 x 10 km, based on a statistically representative method. On each monitoring plot, 30 forest trees were examined for the presence of *Armillaria sp.* (Lushaj *et al.* 1996, 1997; Lushaj 1997, 2000). Altogether, forest trees were investigated in 15 different areas of Albania, belonging to 15 districts. Fruit orchards and vineyards were also investigated in 15 different areas of Albania, belonging to 15 districts.

Field observations and sample collection

The majority of collections were made at middle and high altitude coniferous forests consisting of Silver fir (*Abies alba* L.), Greek fir (*A. borisi-regis* Malf.), Austrian black pine (*Pinus nigra* Arn.), Scots pine (*P. sylvestris* L.), Norway spruce (*Picea abies* (L.) Karst.), etc.

Deciduous forests, consisting mainly of several oak species, were examined at low and lower altitudes, while beech (*Fagus sylvatica* L.) was examined at higher altitudes.

Hop hornbeam (*Ostria carpinifolia* Scop.), Aleppo pine (*Pinus halepensis* Mill.), poplar species (*Populus* spp.), maritime pine (*Pinus pinaster* L.) and eucalyptus species (*Eucalyptus* spp.) were also growing in these forests.

At the same time, the majority of collections were made at low and middle altitudes in fruit orchards and vineyards.

Collections were made from a variety of hosts in these forests as well as in fruit orchards and vineyards. Dead or unhealthy trees, wind-broken trees and cut trees were excavated, and the root collar and a number of roots were inspected for the presence of the characteristic mycelial mats of *Armillaria* under the bark and/or rhizomorphs on the roots. Samples of decayed wood tree bark and rhizomorphs were collected. The presence of basidiocarps was noted and it was recorded whether they were associated with dead or living trees, stumps or wood debris in the ground. The location and altitude were recorded and notes were taken on the condition of the host, with reference to apparent pathogenicity of the fungus and presence of obvious stress factors (Blodgett and Worrall 1992).

During the study years, over 2500 living or dead trees and 600 stumps were investigated in forests, fruit orchards and vineyards of Albania.

Culture media and incubation conditions

Isolations from basidiospores, wood samples and rhizomorphs were made on potato dextrose agar (PDA) amended with fungicides and antibiotics, on malt extract agar 3% (MEA) and on carrot agar (CA) in Petri plates (Intini and Gabucci 1987; Guillaumin *et al.* 1989). Morphological studies of diploid cultures were performed on all these culture media. Monosporous isolations were made on 1-2% MEA (Difco, Detroit MI). All the cultures were incubated in darkness at 24°C.

In order to produce basidiocarps *in vitro,* two types of substrates were used in 500 ml Erlenmeyer flasks: (a) about 100 g of small pieces of oak branches (1.5-2.5 cm in diameter and 2-3 cm long) in 150 ml of deionised water; and (b) a complex medium, consisting of 25 g whole grain rise, 15 g of beech or oak sawdust and 230 ml of 0.5% peptone solution in deionised water (Intini 1991; Tirrò 1991). The substrates were autoclaved two times at 121°C for 1 h with 24-h interval. After inoculation with the vegetative mycelia of *Armillaria* species the flasks were incubated in the darkness at 24°C for about 1.5 month. The temperature was then lowered to 15°C and the cultures were exposed to light with a photoperiod of 11-h (Guillaumin *et al.* 1989; Tirrò and Intini 1989; Tirrò 1991; Intini 1993).

Isolations

Small chips from newly exposed wood, mycelial mats or basidiocarps were plated directly on the surface of PDA, CA or MEA medium. Rhizomorphs were surface sterilized in a solution of NaOCl and 20% ethanol for 2-3 min., then rinsed with sterile water and plated on PDA, CA and MEA (Blodgett and Worrall 1992).

Monosporous cultures were isolated both from basidiocarps collected in the field and from those developed in vitro. Sporulating caps were placed on the lid of a plastic Petri plate (9 cm), in which an opening had been made with a hot surgical blade. Basidiospores were allowed to settle on the surface of the MEA through this opening. After incubation for 24 h, single germinated spores were picked up under a microscope and transferred to a fresh MEA medium with a modified Pasteur pipette (Korhonen and Hintikka 1980).

Pairings

Haploid and diploid isolates were identified in compatibility tests with known haploid tester strains of the European *Armillaria* species (Korhonen 1978; Guillaumin *et al.* 1991). In total, about 40 haploid testers were used in this study; most of them originated from Italy and other European countries, some were from Albania. Each unknown isolate was paired with at least three different tester strains of each *Armillaria* species. Pairings were performed by placing two inocula 1-2 mm apart on the surface of the medium (two pairings per plate). Inocula were taken from the margin of a growing culture with the aid of a modified Pasteur pipette (Korhonen and Hintikka 1980). These inocula were cylinders of agar medium approximately 1 mm in diameter and 2 mm in length. They contained mainly submerged mycelium without crust or rhizomorphs and little aerial mycelium (in initial experiments where the diploid inocula with the dense crustose mycelium were used the mating reaction was not always clear).

In haploid-haploid pairings, 3-4 weeks of incubation were sufficient for the observation of the mating reaction, while in the diploid-haploid pairings the time of incubation was prolonged sometimes up to 6 weeks or more (Guillaumin *et al.* 1991). Before mating tests, diploid cultures of many isolates were preliminarily identified on the basis of culture morphology, so the number of tester strains in diploid-haploid pairings could be reduced to two or three *Armillaria* species.

In case the results were not clear in diploid-haploid confrontations, the pairings were repeated using new tester strains (Guillaumin *et al.* 1991). Also, in some of these pairings, two to three plugs were taken from the side of the haploid testers (3-5 mm past the confrontation line) and they were subcultured in a plate together with the haploid tester. The morphology of these two subcultures, in relation to the reduction in the amount of aerial mycelium, was compared after 2 weeks incubation (Rizzo and Harrington 1992).

RESULTS

Field observations

The surveys carried out during the study years revealed that the average frequency of *Armillaria* disease was 4% for forests and 6% for orchards. In forests of fir, beech, chestnut, oaks, pine and poplar, the infection frequency was up to 15%, and the incidence of damage (decline) was ranked to the first class (Lushaj *et al.* 1996, 1997, 1998; Lushaj 1997, 2000, 2001a, b).

Fructification in vitro

All the five Albanian *Armillaria* species, *A. mellea*, *A.gallica*, *A. ostoyae*, *A.cepistipes* and *A. tabescens*, produced basidiocarps and some of them only primordia, *in vitro*. Basidiocarps were produced on both types of media, but fruiting was more frequent in the complex medium that contained rice, sawdust and peptone. All isolates of *A. tabescens* and A. *ostoyae* fruited on both types of media, but only 31% of A. *cepistipes*, 26% of *A. gallica* and 18% of *A. mellea* isolates fruited, and the fruiting took place only on the complex medium. Basidiocarps developed usually 1-2 months after the cultures were exposed to light, but certain cultures fruited only after 4-7 months incubation in light conditions.

Species identification

A number of collections were identified in the field on the basis of basidiocarp morphology. Basidiocarps were usually observed in October, November and early December. However, the level of fruiting of *Armillaria* was low during most years of survey. An abundance of basidiocarps was observed in certain areas of Albania during October-November in 1996 and 1997, as a result of early rains.

The majorities of Armillaria cultures were isolated from vegetative organs and were considered diploid. Therefore, the species identification was mainly based on diploid-haploid pairings. When monosporous isolates were available, either from natural or cultivated fruit bodies, they were identified in haploid - haploid pairings. In most cases, these pairings verified the species identification that was based on the morphology of the basidiocarps. Identification of most *Armillaria* isolates was also possible on the basis of morphological characteristics of diploid isolates on MEA, CA and PDA culture media (Fig. 1).

In total, 576 *Armillaria* isolates were identified from 43 different host species (Tables 1 and 2). Of them, 397 were diploid isolates, which were identified, in diploid - haploid pairings. Twenty-eight of these diploid isolates produced basidiocarps in vitro and their identification was verified from basidiocarp morphology and haploid-haploid pairings. Monosporous cultures were isolated and identified from 88 basidiocarp collections found in the field.

Of all the isolates identified, 263 belonged to *A. mellea*, 135 to *A. gallica*, 69 to A. *ostoyae*, 49 to *A. cepistipes* and 60 to *A. tabescens* (Tables 1 and 2).

Geographical and latitudinal distribution

Armillaria mellea sensu stricto was present in most of the areas surveyed, but majority of the records comes from northern and central Albania (data available from the author). The species occurred in conifer and in particular in broad-leaved forests at altitudes ranging from 0 m to 1400 m. It was quite common on the oak species (*Quercus* spp.) and on fir species (*Abies* spp.) at altitudes greater than 1100 m. However, in northern Albania the fungus becomes rare at high altitudes; there it was recorded only at altitudes less than 1200 m. *A. mellea* was also found in fruit orchards and vineyards throughout Albania from north to south, at altitudes up 800 m.

A. gallica was less common than *A. mellea* in the forests of Albania, especially in the southern parts, but it was more frequent in the coniferous and broad-leaved forests of the northern and central parts of the country. The fungus was most common at altitudes above 450 m, and occurred up to 1800 m. At low altitudes *A. gallica* was rare, although it was occasionally detected at the elevation close to sea level. *A. gallica* was found in fruit orchards and vineyards only in two areas, one of them in the south (Korçe district) and the other one in the north (Diber district).

A. ostoyae was found everywhere in Albania but is common only in the northern part of the country. It occurred mainly in coniferous forests at altitudes higher than 1200 m and was more frequent at elevations above 1200 m. *A. ostoyae* was not found in fruit orchards and vineyards.

A. cepistipes was found only on mountainous forest areas of northern and central Albania, in beech forests at elevations from 800 to 1600 m, in coniferous forests of pine, fir or spruce and in mixed forests at altitudes ranging from 800 m to 1800 m. *A. cepistipes* was not found in fruit orchards and vineyards.

A. tabescens was least commonly recorded of all *Armillaria* species. It was found predominantly on oak, sometimes on fir, poplar, eucalyptus etc. It occurred in oak forests at altitudes from 400 m to 900 m, in fir forests at altitudes from 1000 m to 1300 m, in poplar stands at altitudes from 100 m to 300 m and on eucalyptus at altitudes from 0 m to 200 m. The fungus also attacked almond orchards in two different localities: in the Diber district, northern Albania, and in Korçe, southern Albania.

Ecology and parasitic behavior of Armillaria species

Conifers

Fir species. All five *Armillaria* species were recorded from fir forests (Table 1). *A. mellea* and A. *ostoyae* cause significant losses, killing trees of all ages, single trees or small groups of 3-10 trees.

- *A. mellea* was the predominant species on *Abies alba* in northern Albania (Table 1). In mixed coniferous forests of pine, spruce, and fir at elevations from 600 m to 1000 m, *A. mellea* was killing fir trees selectively, while mortality of oak or pine was rare. Infection of fir by *A. mellea* was also quite common at higher altitudes up to 1400 m. Basidiocarps of this fungus were usually associated with dead trees or stumps, but they were also detected at the base of living trees, some of them with no apparent symptoms in the crown. - *A. ostoyae* was very common in *Abies alba* forests in northern and central Albania and in *Abies borisi-regis* in southern Albania. In mixed forests of fir, beech, pine and spruce the fungus was observed to kill selectively fir trees only; no infections were noticed on beech trees. - *A. gallica* was present in most of the fir forests examined, and it was the predominant species in *Abies borisi-regis* forests of southern Albania. It was usually associated with small-suppressed trees in the understory, but was also found on larger dead trees. In some cases *A. gallica* was isolated from firtrees that were infected also by other diseases. - There were nine records of *A. cepistipes* on *Abies alba*. - *A. tabescens* was recorded only three times on fir species, on trees infected also by other diseases and / or insects.

Spruce species. In *Picea abies* forests of Tropoje district, in the northern Albania, the most frequent cause of mortality was *A. ostoyae* while *A. gallica* and *A. cepistipes* were recorded as saprophytes.

Pine species. In *Pinus nigra* forests of northern Albania, *A. ostoyae* was found to cause significant problems. *A. mellea* caused mortality in the reforestation's of this tree species, established 25-30 years ago in cleared broad-leaved (beech and oak) forests in Puka district in northern Albania and in Pogradec and Korçe districts in the southern of Albania. In northern Albania, Tropoje district, *A. ostoyae* killed Scots pine trees. *A. cepistipes* was usually a saprophyte on Scots pine, with two exceptions where the fungus was considered responsible for killing a tree suppressed in the understory. *A. mellea* caused mortality of Aleppo pine forests at altitudes from 100 m to 350 m. The fungus was common in mixed stands of Aleppo pine and deciduous oaks, but only Aleppo pine trees were infected. *A. gallica* was found to cause problems in reforestation of Maritime pine established 35-40 years ago at altitudes from 100 to 300 m.

Other conifers. *Juniperus communis* L., growing in understory of other conifers, was occasionally attacked by *A. mellea* and *A. ostoyae*. These fungi were recorded also from *Cupressus sempervirens* in Berat district, southern Albania.

Deciduous forests

Beech. Many beech forests throughout Albania were investigated in this study. Four *Armillaria* species were detected (Table 1). *A. gallica* was the predominant species. In most cases, it was a saprophyte on stumps and dead wood, producing an abundance of rhizomorphs in the soil and forests litter. However, in some cases, *A. gallica* was found to kill small suppressed trees in the understory of coppice forests, where the fungus had infected the tree through the stump. In high altitude forests, *A. cepistipes* was observed to cause mortality of small suppressed trees, but generally the fungus was a saprophyte. It produced an abundance of rhizomorphs, similar to those of *A. gallica*. *A. mellea* was found in most beech forests. *A. ostoyae* was recorded as a saprophyte in beech forests but in a few cases it was found to cause mortality to young beech trees. *A. tabescens* was not found in the beech forests.

Oak species. Also many oak forests were investigated; *A. mellea*, *A. gallica* and *A. tabescens* were found. These fungi killed isolated young trees or were growing as saprophytes in old stumps. *A. mellea* was the predominant species on all species of oak: *Quercus cerris*, *Q. petrea*, *Q. frainetto*, *Q. pubescens*. *Q. aegilops* and *Q. ilex*. In most cases *A. gallica* was a saprophyte on stumps and dead wood of all species of oak. *A. tabescens* was also found on all oak species, most commonly on *Q. ilex* (Table 1). *A. ostoyae* and *A. cepistipes* were not found on oak.

Other deciduous trees. *A. mellea*, *A. gallica* and *A. tabescens* were found in pure poplar stands (*Populus* spp.). *A. tabescens* was found on *Eucalyptus* species. In the pure deciduous and mixed deciduous-conifers forests of Albania, *A. mellea* infected *Castanea sativa*, *Ostria carpinifolia* and other species, including occasionally *Fraxinus excelsior*, *Carpinus betulus*, *Platanus orientalis* and *Corylus avellana*. *Armillaria gallica* was recorded on *Castanea sativa*, and *A. mellea*, *A. ostoyae* and *A. cepistipes* were found on *Betula pendula* and other species too.

Fruit trees and garden plants

Three *Armillaria* species were found to cause infections of Albania: *A. mellea*, *A. gallica* and A. *tabescens* (Table 2).

A. mellea was the predominant *Armillaria* species on the fruit orchards, vineyards and private gardens, infecting a variety of ornamental plants and trees in different areas. It was found to cause significant damage in the vineyards of every district, especially in areas cleared from oak forests, often together with other diseases (e.g. *Rosellinia* spp.) or insect pests. *A. mellea* was recorded also from lemon tree (*Citrus limon*), ficus (*Ficus carica*), walnut (*Juglands regia*), nerium (*Nerium oleander*), apple trees (*Malus* spp. and on *Malus sylvestris*), mulberry tree (*Morus nigra* L. and *Morus alba* L), olive species (*Olea* spp. and *Olea europaea* L.), pear trees (*Prunus* spp.) and thuja (*Thuja orientalis*).

Infections of *A. gallica* on cultivated plants were recorded in two different areas: in Korçe, southern Albania, and in Diber, northern Albania. The fungus caused mortality on cherry, apple and pear. However, the fungus other was not detected in other areas of southern Albania, and it does not seem to play an important function as a pathogen.

A. tabescens was found to cause significant damage in almond tree orchards in two different localities of Albania, in Korçe and in Diber. Two orchards had been established on former forestland cleared from broad-leaved trees.

According to species, the number of collections was *A. mellea* 277, *A. gallica* 135, *A. ostoyae* 69, *A.tabescent* 62 and *A. cepistipes* 49 (Tables 1 and 2).

Table 1. *Armillaria* records on different host species and altitudes in the forests of Albania.

No.	Host species	Working circles (1)	Altitude in m North Centre South	*A. mellea*	*A. gallica*	*A. ostoyae*	*A. cepistipes*	*A. tabescens*
I	Conifers							
1	Silver fir (*Abies alba* Mill.)	07,13,24,34,41, 42	600-1800 700-1000 700-1300	10	14	14	9	2
2	Greek fir *(Abies borissi-regis Matt.)*	41,42	- - 700-900	2	4	2	-	-
3	Junipers (*Juniperus communis L.*)	37,38,39	- - 100-800	3	-	3	-	-
4	Norway spruces (*Picea abies* (L.) Krast.)	07	900-1800 - -	-	3	3	3	-
5	Serbian spruce (Picea omorica L.)	07	900-1800 - -	-	3	3	3	-
6	Austrian black pine (*Pinus nigra* Arn.),	12,13,28,31,34	400-1200 800-1000 800-1200	2	3	28	-	-
7	Aleppo pines (*Pinus halepansis* Mill.)	20,22	- 100- 300 -	4	-	-	-	-
8	Maritime pine (*Pinups pilaster* Sol.)	20,22	- 100- 300 -	-	2	-	-	-
9	Scots pine (*Pinus sylvestris* L.)	07,13	900-1800 - -	-	-	4	2	-
10	Cypresses (*Cupressus sempervirens L.*)	37,38,39	- - 100-800	3	-	3	-	-
II	Broad-leaved							
11	Birch (*Betula pendula* Roth.*)*	31	- - 800-1200	6	-	6	6	-
12	Hop hornbeam (*Ostria carpinifolia* Scop.)	01	400- 900 -	3	-	-	3	-
13	Hornbeam (*Carpinus*	11	600- 800	1	-	-	-	-

	betulus L.)		- -					
14	Sweet chestnut (*Castanea sativa* Mill.)	02,06,27,29	350-1100 700-900 700-900	14	4	-	-	-
15	Hazelnut (*Corylus avellana* L.)	09	800-1600. - -	1	-	-	-	-
16	Common beech (*Fagus sylvatica* L.)	03,09,10,14,19, 21,23,31	800-1600 900-1100 800-1200	19	24	3	23	-
17	Ash *(Fraxinus excelsior* L.)	17	600-800 - -	1	-	-	-	-
18	Oriental planetree (*Platanus orientalis* L.)	22,26	- 100-400 400-600	1	-	-	-	-
19	Poplar sp. (*Populus spp.*)	37	- - 100-300	3	6	-	-	6
20	Turkey oak (*Quercus cerris* L.)	01,04,05,08,11, 12,15,16,17,18, 25, 38	250-900 300-900 300-900	26	24	-	-	6
21	Hungarian oak (*Quercus frainetto* Ten.)	11,12,15,16,17, 18, 38,	250-900 300-900 300-900	16	11	-	-	6
22	Sessile oak (*Quercus petrea* (Matt) Liebl.)	01,04,05,12,15, 16,17,18,25,30	250-900 300-900 300-900	37	11	-	-	6
23	Pubescent (Downy) oak (*Quercus pubescens* Willd.),	01,04,05,12,15, 16,17,18,25,28, 30, 39	250-900 300-900 300-900	31	6	-	-	3
24	Aegilops oak (*Quercus aegilops* L.)	35	- - 0-200	8	6	-	-	6
25	Holm oak (*Quercus ilex* L.)	32,33,35	- - 0-600	6	6	-	-	9
26	Eucalyptus sp. (*Eucaliptus spp.*)	36,40	- - 0-200	-	-	-	-	9
	Total	42		198	127	69	49	53

Number indicate the working circles (forest economy):
1, Shkoder (Rosek-Drisht); 2, Shkoder (Shllak 1); 3, Shkoder (Shllak 2); 4, Has (Tregtan); 5, Has (Perollaj); 6, Tropoje (Geshtenjat e Tropojes); 7, Tropoje (Valbone-Dragobi); 8,Tropoje (Bytyç); 9, Tropoje (Nikaj-Mertur); 10, Tropoje (Kolgecaj); 11, Puke (Dedaj-Bahot); 12, Puke (Puke-Fushe Ares); 13 Puke (Tuç); 14, Puke (Munelle); 15, Mirdite (Kulmet e Dervenit); 16, Mirdite (Shtane); 17, Mirdite (Lugina e Fanit te Madh); 18, Mat (Derjan 1); 19, Kruje (Qafe Shtame); 20, Kruje (Kraste); 21, Tirane (Bize); 22, Tirane (Parku i Tiranes); 23, Librazhd (Lepushe); 24, Librazhd (Qarrishte); 25, Librazhd (Drogostuje); 26, Librazhd (Librazhd); 27, Pogradec (Geshtenjat e Pogradecit); 28, Pogradec (Verdove); 29, Pogradec (Stropske); 30, Korçe (Gorice); 31, Korçe (Dardhe); 32, Vlore (Maja e Bratit); 33, Vlore (Gumenice); 34, Vlore (Llagara);35, Vlore (Himare); 36, Vlore (Kume-Shashice); 37, Berat (Hija e Roshnikut); 38, Berat (Mali i Bardhe); 39, Berat (Mali Partizan); 40, Fier (Lumi Seman-Rosk); 41, Gjirokaster (Bredhi i Sotires 1) and 42, Gjirokaster (Bredhi i Sotires 2).

Table 2. *Armillaria* records on different cultivated host species and altitudes in the fruit trees of Albania.

No.	Host species	Altitude in m North Centre South	Districts (2)	*A. mellea*	*A. gallica*	*A. ostoyae*	*A. cepistipes*	*A. tabescens*
1	Lemon *(Citrus limonia* Osbeck.*)*	- - 0-200	e,k	2	-	-	-	-
2	Fig-tree *(Ficus carica* L.)	- 0-500 0-500	b,d,m,n,i,g,h,o	6	-	-	-	-
3	Walnut- tree *(Juglands regia* L.)	350-1100 - -	a,f	2	-	-	-	-
4	Wild apple-tree (*Malus silvestris (L.) Mill.)*	350-800 350-800 350-800	a,f,m,l	4	-	-	-	-
5	Apple- tree sp. (*Malus spp.)*	350-800 350-800 350-800	a,f,l,m,n,	5	2	-	-	-
6	White Mulberry- tree (*Morus alba L.)*	100-600 100-600 100-800	a,f,m,n,l,	5	-	-	-	-
7	Black Mulberry-tree (*Morus nigra L.)*	100-600 100-600 100-800	a,f,m,n,l,	5	-	-	-	-
8	Oleander (*Nerium oleander* L.)	- 0-200 -	d	1	-	-	-	-
9	*Olive-tree (Olea europaea* L.*)*	- 0-400 0-400	c,ç,e,n.g,h,i,k	9	-	-	-	-
10	Olive-tree sp. *(Olea spp.)*	- 0-400 0-400	c,ç,e n.g,h,i,k	9		-	-	-
11	Plum-tree *(Prunus avium l.)*	350-900 350.900 350-900	f,l	2	4	-	-	-
12	Plum-tree *(Prunus domestica* L.*)*	350-900 350.900 350-900	f,l	2	-	-	-	-
13	Plum-tree sp. *(Prunus spp.)*		f,l	2	-	-	-	-
14	Plum-tree (*Prunus dulcis* L.*)*	350-900 350.900 350-900	f,l	2	1	-	-	6
15	Plum-tree (*Prunus persica* L.*)*	350-900 350.900 350-900	f,l	2	1	-	-	1
16	Pear–tree sp. (*Pyrus spp.*	350-900 350.900 350-900	f,l,m	3	-	-	-	1
17	Wild pear –tree (*Pyrus communis* L.*)*	350-900 350.900 350-900	f,l,m	3	-	-	-	1
18	Wall- Creeper *(Thuja orientalis* L.)	- 0-200	d	1	-	-	-	-
19	Vine *(Vitis*	350-800	f,l,m,n	4	-	-	-	-

	vinifera L.)	100-800						
		0-800						
20	Vineyard (*vine spp.*)	350-800	f,l,m,n	4	-	-	-	-
		100-800						
		0-800						
21	Peach- tree (*Persica*	350-900	f,l,m	3	-	-	-	-
	vulgaris Mill.)	350-900						
		350-900						
	Total		15	79	8	-	-	9

(2) Letters indicate districts in which collection were made from agricultural plants and vineyards:

A, Tropoje; b, Lezhe; c, Lushnje; ç, Fier; d, Tirane; e, Vlore; f Diber; g, Mallakaster; h, Durres; k, Sarande; i, Elbasan; l, Korçe; m, Shkoder; n, Berat; o, Kruje.

DISCUSSION

Albania is a mountainous country and in spite of its small size the landscapes and climatical conditions are variable (Lushaj *et al.* 1996, 1997). The climate on the coastal area is of Mediterranean type with hot and dry summers and mild and rainy winters. In inland there is a transition from the maritime climate to a continental and mountainous climate, with more abundant rainfall. Covering 28 749 km^2 and located at the same latitude as central and southern Italy (Lushaj 2001a), Albania presents a great variety of plant and fungal flora.

Five *Armillaria* species were identified in a nation-wide survey in Albania. They include all the European *Armillaria* except *A. borealis* and *A. ectypa*.

Armillaria mellea sensu stricto is the most common species in Albania and the most significant pathogen in forest and cultivated plants. It was recorded from several conifers and broad-leaved trees in most areas examined, except the high altitudes above 1100-1200 m of the forest of the north of Albania. The fungus was mostly a weak parasite or a saprophyte of forest trees, but it also causes significant damage on *Abies* spp., *Quercus* spp., *Fagus sylvatica*, *Castanea sativa*, *Populus* spp., *Ostria carpinifolia* etc. Moreover, *A. mellea* was commonly found on cultivated plants as well in fruit orchards, causing damage especially on apple and pear species and in vineyards.

The fungus is considered thermophilic, and in most areas of central Europe it is restricted to low altitudes. In France, *A. mellea* has not been reported at altitudes greater than 1000 m (Guillaumin *et al.* 1993), but in southern Italy, it has been found at altitudes up to 1400 m (Intini 1991; Grillo *et al.* 1996). In Greece it was present in most areas examined, except high altitudes (above 1100 m) of northern Greece (Tsopelas 1999). The situation was about the same in Slovenia (Munda 1997) and in Hungary (Szanto 1998).

In most areas investigated in Europe and North America, *A. mellea sensu stricto* occurs mainly on broad-leaved forests and is rare in coniferous forests (Kile *et al.* 1991; Legrand and Guillaumin 1993). In Albania, however, *A. mellea* is the predominating *Armillaria* species in *Abies alba* forests, causing considerable damage.

Armillaria gallica is a weak parasite or a saprophyte of mostly deciduous trees, occurring in central and southern Europe. Also, in Albania, it is rare on conifers but common on broad-leaved trees. At higher altitudes (ca. 600-1600 m) it is found commonly on beech, chestnut, etc. At lower altitudes (0-600 m), the main host trees are several species of *Quercus*. Occasionally it was found on cultivated plants as apple and pear species at altitudes from 350 to 900 m.

A. ostoyae is rare in central and southern Albania, but it is common in the forests of the northern Albania and causes significant damage on *Pinus nigra, P. sylvestris, Picea abies, P. omorica, Abies alba*, etc., at altitudes from 600 to 1800 m. At lower altitudes (ca. 100-800 m), *A. ostoyae* was recorded on *Pinus pinaster*, *Cupressus sempervirens* and *Juniperus communis*. *A. ostoyae* was not found in fruit orchards and vineyards.

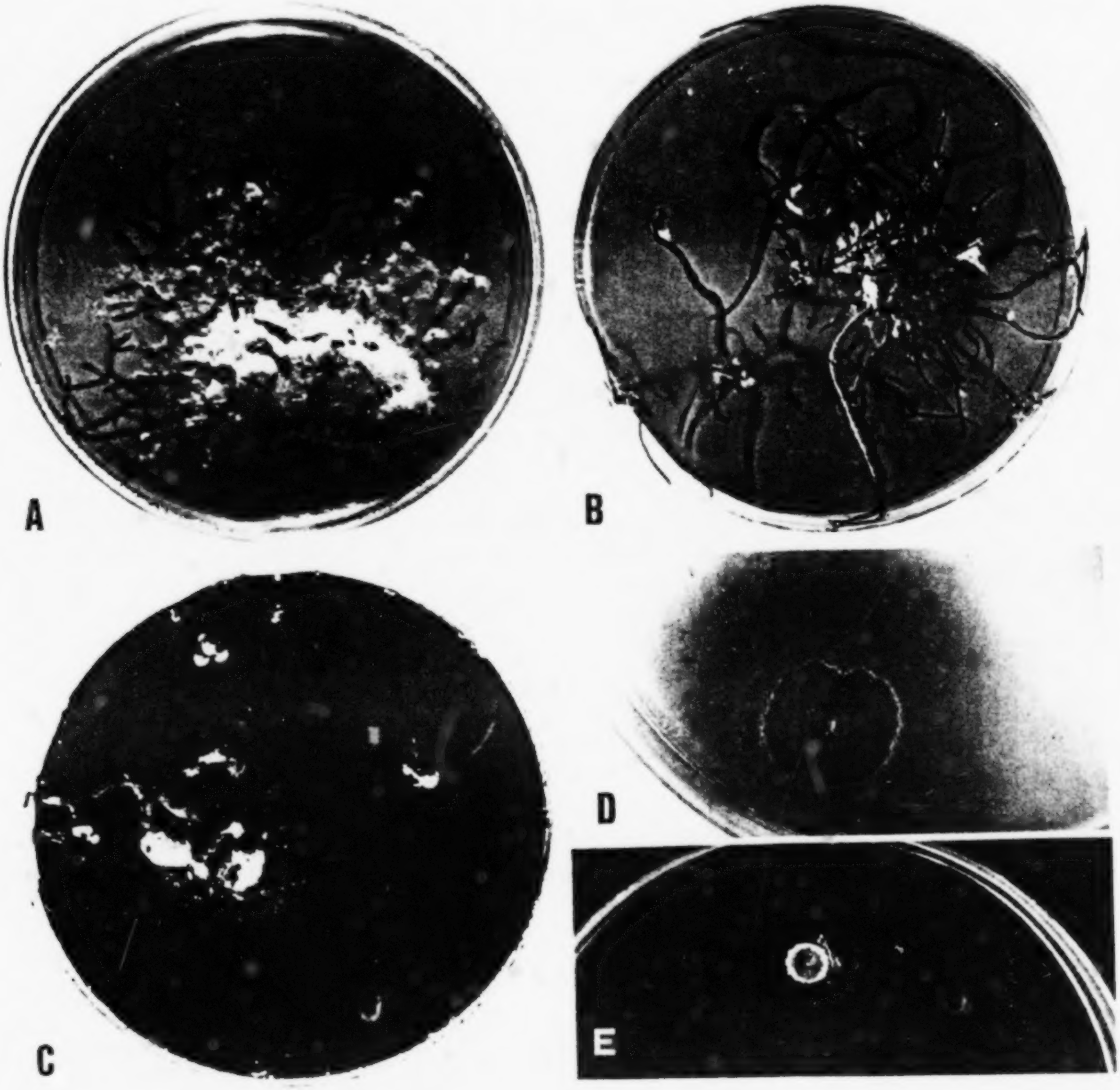
In coniferous forests of Europe and North America, *Armillaria ostoyae* is the most important pathogen among the *Armillaria* species (Kile *et* al. 1991). In areas of central Europe with a continental type of climates, the fungus occurs in the forests independently of altitude, while in zones of Mediterranean climate is found only at high altitudes (Guillaumin *et* al. 1993). Albania is on the southernmost limit of this fungus in the Balkan region but in Italy *A. ostoyae* has been found as far south as in Calabria (Intini 1996; Grillo *et* al. 1996).

Armillaria cepistipes was recorded in northern Albania at high altitudes from 800 to 1800 m's' and in southern Albania at altitudes from 800 -1200 m, mostly as a saprophyte on conifers and broad-leaved trees, predominating in beech and silver fir forests. It was not found in fruit orchards and vineyards. The species has a northern distribution but along the mountain ranges its distribution in southern Europe extends to Calabria, southernmost Italy (Intini 1996; Grillo *et* al. 1996).

Armillaria tabescens was found commonly on several species of oak, *Populus* spp., *Eucalyptus* spp., etc. Among cultivated plants, it was found to cause disease in almond trees as well as on pear trees (*Prunus* spp.).

At the moment, it is difficult to talk about the economical importance of *Armillaria* in Albania, as well as the need and possibilities to control them, because we do not have the data. It is an obligation for the near future.

Figure 1. Morphological characteristics of the diploid mycelia of the five *Armillaria* species present in Albania. The cultures were grown on 3% malt extract agar for 3 weeks. A) *A. mellea*: white cottonous colony, flat rhizomorphs with irregular branches, scarce pigmentation around the rhizomorphs. B) *A. gallica*: rhizomorphs circular in cross section, slight pigmentation around the rhizomorphs. C) *A. cepistipes*: brown colony with scarce rhizomorphs, circular in cross section, considerable pigmentation around the rhizomorphs. D) *A. ostoyae*: crustose colony with scarce rhizomorphs, brown pigmentation around the colony. E) *A. tabescens*: scarce mycelium without rhizomorphs and pigmentation.



IMPACT OF ARMILLARIA rRNA – IGS GROUPS ON CROWN CONDITION OF MAPLES IN PORTNEUF COUNTY

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ABSTRACT

Portneuf County, located along the main pollutant transport line from the American Midwest and the Great Lakes, is associated with industrial development. Eighteen plots, located in the southern part of the county, were assessed from 1992 to 1997 for sugar maple health, including Armillaria root rot, and for pollutants in throughfall. One hundred and forty-five rhizomorphs were collected on plot trees and genotyped using rRNA-IGS. Fifty reliably identified voucher collections were genotyped as controls. Rhizomorphs were identified as follows: *A. calvescens* (29 samples), *A. sinapina* (47 samples), a third group sharing *A. calvescens – A. gallica* NA and *A. gallica* Eur. patterns (33 samples). Ninety-one samples remained undetermined. Contingency tables were analysed for the effect of Armillaria groups on sugar maple condition, controlling for the pollution class and canker/wound status, using Mantel-Haenszel's mean score statistic. Trees without Armillaria rhizomorphs had 2.3 times more chances of being healthy than those with rhizomorphs. *A. sinapina* was associated with a higher damage level than *A. calvescens*.

Keywords: *Armillaria calvescens*, *Armillaria sinapina*, IGS genotypes, throughfall, sugar maple crown condition

INTRODUCTION

Sugar maple decline in the early 1980's triggered many research projects. Numerous causes have been suggested, including acid rain, air pollutants, drought, late frost, insect damage and defoliation and Armillaria root rot (Allen et al. 1992; Bauce and Allen 1992; Bernier et al. 1989; Brodeur and Maufette 1989; Gagnon 1986; Lachance 1985, 1989; Roy et al. 1985). The extent of decline in the Portneuf County area reached 60 000 ha in 1986 (Gagnon 1987; Gagnon and Bordeleau 1990).

Portneuf County is located west of Quebec City, on the north shore of the St. Lawrence River, along the main pollutant transport line from the American Midwest and the Great Lakes (Boulet 1990). It has six industrial parks and 87 smaller industrial areas, covering 2 080 ha (J. Landry, MRC de Portneuf, personal communication).

Following the implementation of the Canadian government's Green Plan (Environnement Canada 1990), a study on sugar maple health related to local and long distance transported pollutants was implemented in Portneuf County. The aim of this paper is to report on the impact of Armillaria species on the health of maple stands in Portneuf County in relation to air pollution and other woody tissue damage.

MATERIALS AND METHODS

Network design

The Portneuf County maple study was initiated in 1991 and 1992. Initially, the network included 20 plots distributed along three transect lines (Figure 1). The project ended in 1996, but crown condition was rated in 1997 for 18 plots out of 19.

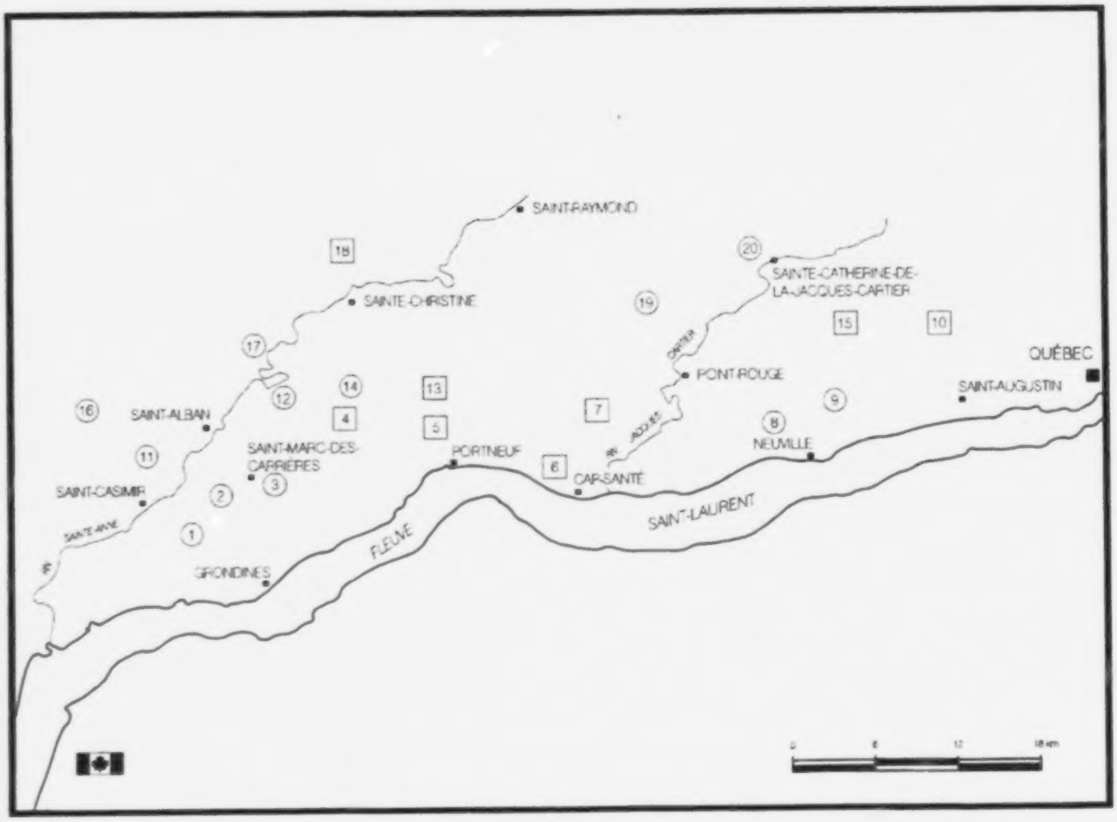
SAINT-RAYMOND
18
20
SAINTE-CATHERINE-DE-LA-JACQUES-CARTIER
SAINTE-CHRISTINE
19
15
10
17
CARTIER
QUÉBEC
PONT-ROUGE
14
13
SAINT-AUGUSTIN
16
SAINT-ALBAN
12
4
7
9
5
JACQUES
8
11
SAINT-MARC-DES-CARRIÈRES
PORTNEUF
NEUVILLE
3
6
CAP-SANTÉ
SAINT-CASIMIR
2
SAINT-LAURENT
SAINTE-ANNE
1
FLEUVE
GRONDINES
0
6
12
18 km

Figure 1. The Portneuf County Green Plan study on sugar maple health related to local and long distance transported pollutants: location of plots established in 1991 (square) or 1992 (circle).

Transect lines were established as follows: the south line along a quarter of the distance between the St. Lawrence River shore and the Boreal Shield foothills; the middle line along three quarters of the distance between the St. Lawrence River shore and the Boreal Shield foothills; the northern line in the middle of the slope of the first Boreal Shield mountains. Study plots were established at an approximate distance of 5 km along the southern transect and 10 km on each of the other two lines. Plots had to be 1 km away from any major industry or highway. As no sugar maple stands were found between plot 13 and plot 15, plot 14 was alternately located in the middle of the county. One plot was lost in 1993 (plot 17, Saint-Alban). Each plot consisted of three circular subplots randomly located in the selected maple stands. Subplot radius varied from 10 to 21 m in order to include 20 sugar maples (*Acer saccharum* Marsh.) in each subplot.

Health rating

Tree health was assessed according to the ARNEWS methodology (Magasi 1988; D'Eon et al. 1993). Measurements (ARNEWS form 4) included crown condition; current, insect and disease defoliations; abiotic symptoms; woody tissue damage, including open wounds, cankers and root rots. Only crown condition was rated in 1997 on 18 plots out of 19. Crown condition ratings for live hardwoods ranged from 10 (perfect tree) to 70 (moribund tree), according to the percentage of dead branches and/or bare twigs. Sugar maple was assessed from 1991-92 through 1997.

An additional rating of open wounds and cankers was performed in 1993-94. Canker and open wounds, including maple borer galleries, were assessed on each tree. Non-destructive assessment of Amillaria infection was performed in 1993-94 and reassessed in 1996. Mosses, litter fall and exfoliating dead bark layers were delicately pulled away from superficial roots and root flare without damaging the trees. Rhizomorphs penetrating between bark cracks or layers were recorded (Roy et al. 1985) and were sampled whenever possible. Sixty sugar maples had Armillaria rhizomorphs but could not be sampled.

DNA analysis for *Armillaria* spp. genotyping

Genomic DNA from rhizomorphs from 145 sugar maples in the Portneuf network and 50 *Armillaria* spp. voucher collections, consisting of fruiting bodies, was PCR-amplified with specific primers of the intergenic spacer of the nuclear DNA coding for the ribosomal gene (rRNA-IGS) according to Harrington and Wingfield (1995). Forty-eight voucher collections, which were used as controls, had been previously identified by crossing with tester strains by Bérubé (Bérubé and Dessureault 1988, 1989) and Morrison (Canadian Forest Service, Pacific Forestry Centre, pers. comm.). Two other specimens from plot 2 were identified while fresh by their distinctive morphological characters. PCR products were digested with *Alu*I and analysed by electrophoresis in agarose gels in order to genotype Armillaria samples by comparison with already reported digestion patterns (Harrington and Wingfield 1995). Since *Alu*I produced a single restriction pattern for *A. ostoyae*, *A. gemina*, additional restriction assays were performed with *Nde*I, *Bsm*I, or *Hind*II.

Throughfall sampling and analysis

Throughfall water was sampled in all 19 plots at 12 sampling periods from 1992 to 1996: two collections were performed in 1992, three each in 1993, 1994 and 1996 and one in 1995. Four samples $plot^{-1}$ $period^{-1}$ were collected in 1992 and 1993 randomly distributed in subplot 1 and six samples $plot^{-1}$ $period^{-1}$ were collected in 1994-96. Two bottles were randomly distributed in each subplot in 1994-96. Mean sampling duration was 14.5 days (min. 10 days; max. 19 days). Samples were collected between mid-June and the end of September in 1 L opaque bottles with a 10 cm diameter funnel slightly plugged with mesh cloth to exclude solid material.

Sample volumes were measured and samples were filtered (0.45 µm) and kept at 4-6°C for a short period before analysis. Sample acidity was measured with a pHmeter (radiometer PHM82). Samples were analysed for K^+ and Na^+ by atomic emission spectrophotometry; for Ca^{2+} and Mg^{2+} atomic absorption spectrophotometry; for NH_4^+ by colorimetry; for Cl^-, F^-, NO_3^- and SO_4^{2-} concentrations by ionic chromatography (Dionex), in 1992-94 (Boutin and Robitaille 1995). Analyses were performed by ICP in 1995 and 1996. Fluorine was not analysed in collections 6 and 7 (1994). H^+ concentrations were calculated from pH measurements. Total bottle content for one ion was obtained by multiplying the collection volume by the ion concentration. Mean sample content was then calculated for H^+, F^-, NO_3^- and SO_4^{2-} for each plot. Some samples were excluded from the means calculation: samples with volumes exceeding 1000 mL, with very low volumes (10-20 mL), with bottle or funnel tipped or with signs of contamination by droppings, such as abnormally high K^+ or NH_4^+ concentrations. The minimal and maximal numbers of samples per mean were 50 and 60 respectively (37 and 48 for F^-).

Each plot was characterised as "low" (below or equal to the median content) or "high" (above the median content) for each of the four following pollutants: H^+, F^-, NO_3^-, SO_4^{2-}. Plots with three or four high scores were rated "high" in the overall pollution class; others were rated as "low" (Table 1).

Table 1. Fluorine (F^-), nitrate (NO_3^-), sulphate ($SO_4^{\ 2-}$), acidity (H^+) and global pollution index by plot in the Portneuf network.

Plot	F+	NO_3^-	SO_4^{2-}	H^+	Global index
1	Low	Low	Low	Low	Low
2	Low	Low	High	Low	Low
3	High	High	High	Low	High
4	Low	High	High	High	High
5	High	High	High	High	High
6	High	Low	High	High	High
7	Low	High	High	Low	Low
8	Low	Low	Low	Low	Low
9	Low	Low	Low	Low	Low
10	Low	High	Low	Low	Low
11	Low	Low	Low	Low	Low
12	High	High	Low	High	High
13	High	High	High	High	High
14	Low	High	Low	High	Low
15	Low	Low	Low	Low	Low
16	High	Low	High	High	High
18	High	Low	High	High	High
20	High	Low	Low	High	Low

Statistical analysis

Trees were given an overall tree condition rating following the crown condition assessments from 1993 to 1997 (Table 2). Contingency tables were built from the overall pollution class (low - high) by the canker and/or wound class (absent - present) by the Armillaria group (5 groups) by the overall tree condition (3 levels, Table 2).

Table 2. Overall tree condition from 5-year observation of annual crown conditions (ARNEWS methodology) in the Portneuf network.

Tree condition	Description	Related annual crown condition codes
1	Healthy	4 or 5 years with crown condition code 35 or less and no code 50 or more
2	Affected	2 or 3 years with codes 40 or 45 and no code 50 or more
3	Declining or dead	At least one year with code 50 or more or 4-5 years with code 40 or 45 or tree dead in 1997

Armillaria specimens from Portneuf were grouped according to their genotype identification into five different groups. Group 0 was for absence of Armillaria; group 1, for *Armillaria calvescens* Bérubé & Dessureault; group 2 for a group with a mixed pattern from *A. calvescens* and *Armillaria gallica* Maxmüller & Romagnesi (both European (Eur.) and North American (NA)); group 3 for *Armillaria sinapina* Bérubé & Dessureault and group 4 was for unidentified or unsampled *Armillaria* spp. or genotypes with only one sample.

The 2 x 2 x 5 x 3 contingency table was analysed for effect of Armillaria group on tree condition, controlling for the pollution class and canker/wound status, using the mean score statistic from the general Mantel-Haenszel methodology with SAS FREQ procedure (Stokes et al. 1995). The mean score for each group has been calculated from the tree condition value (table 2), which is ordinal. Two additional 2 x 2 x 2 x 2 tables were built as follows: pollution class (high – low) by canker/wound class by Armillaria (presence or absence) by tree condition (1: healthy versus others; 2: affected versus very affected, declining or dead). These tables were used to calculate the odds of a tree being healthy (affected in the second additional table) without any Armillaria infection, as compared with Armillaria infection, controlling for pollution and canker/wound status (Stokes et al. 1995), using the SAS FREQ procedure.

RESULTS AND DISCUSSION

Armillaria genotypes

The results of rRNA-IGS identification of voucher specimens are presented in Table 3. All *Armillaria gemina* Bérubé & Dessureault were identified as such by rRNA-IGS genotyping. Most *Armillaria ostoyae* (Romagnesi) Herink (28/31) were identified as such by their IGS pattern. Five out of six *A. calvescens* specimens displayed the common *A. calvescens – A. gallica* NA pattern (Harrington and Wingfield 1995), the sixth one showing *A. gallica* Eur. pattern. The only *Armillaria mellea* (Vahl:Fr.) Kummer specimen was genotyped as *A. ostoyae*. The inadequate rRNA-IGS genotyping of these two voucher specimens shows the limits of this method. Three eastern *A. sinapina*, from conifers or unidentified wood debris (QFB 7726, 7754 and 7755), were genotyped as *A. sinapina*. The *A. sinapina* fruiting bodies from hardwoods (QFB 7756, 7757, 7758, 16744, 16747) showed *A. gallica* Eur. pattern. As *A. gallica* Eur. does not exist in North America, rhizomorphs bearing *A. gallica* Eur. patterns will be from now on considered as *A. sinapina*. Western collections of *A. sinapina* exhibited the *Armillaria nabsnona* Volk & Burdsall rRNA-IGS pattern.

Table 3. *Armillaria* spp. voucher collections: identification by tester strain or distinctive morphological characters compared with rRNA-IGS genomic identification.

Identification by			
Tester strains	**rRNA-IGS**	**QFB[1] No.**	**Total**
A. calvescens	*A. gallica* Eur.	7753	1
A. calvescens	*A. calvescens / A. gallica* NA	7748, 7749, 7750, 7751, 7752	5
A. gemina	*A. gemina*	7753, 7745	2
A. mellea	*A. ostoyae*	7727	1
A. ostoyae[2]	*A. ostoyae*	7706 to 7722, 7729, 7731, 7732, 7735, 7737 to 7742, 7931	28
A. ostoyae	*A. calvescens / A. gallica* NA	7713A	1
A. ostoyae	No identity	7729, 7736	2
A. sinapina (east[3])	*A. sinapina*	7726, 7754 to 7758, 16744[5], 16747[5]	8
A. sinapina (west[4])	*A. nabsnona*	7733, 7734	2

1: From the René-Pomerleau Herbarium, Natural Resources Canada, Canadian Forest Service, Sainte-Foy, Québec; 2: includes three specimens from British Columbia, from D. Morrison; 3: specimens from eastern Canada; 4: specimens from British Columbia; 5: specimens identified from distinctive morphological characters, while fresh.

From the 145 Portneuf maple rhizomorphs analysed, 29 displayed the *A. calvescens – A. gallica* NA pattern. *A. calvescens* and *A. gallica* NA are known to be related (Miller et al. 1994, Smith and Anderson 1989) and give the same *Alu*I pattern (Harrington and Wingfield 1995). The rRNA-IGS pattern obtained from *A. calvescens* voucher specimens, the specificity of *A. gallica* for oak in North America (Blodgett and Worrall 1992a, b) and the preference of *A. calvescens* for maple trees and stands (Blodgett and Worrall 1992a; Sabourin et al. 1990) strongly suggest that rhizomorphs showing *A. calvescens – A. gallica* NA pattern are from *A. calvescens*.

Thirty-three samples had a mixed pattern between *A. calvescens* – *A. gallica* NA and *A. gallica* Eur. patterns. The status of rhizomorphs exhibiting such a mixed pattern remains unclear.

Forty-two samples displayed the *A. sinapina* pattern; five samples displayed a mixed pattern between *A. sinapina* and *Armillaria cepistipes* Velenovsky, but, as *A. cepistipes* has never been found in eastern North America, these are considered to be *A. sinapina*. One sample showed the *A. ostoyae* pattern and 95 could not be sampled or genotyped.

Sugar maple health status

The impact of Armillaria species on the overall tree condition is summarised in Table 4. Proportions of healthy trees are higher when no Armillaria has been detected (35.7%) and decrease with the presence of *A. calvescens* (24.2%) and even more with *A. sinapina* (12.8%), while the number of very affected, declining or dead trees increases from 24.9% without Armillaria to 44.8% *A. sinapina* infection. The Armillaria group mean scores differ significantly ($P<0.0001$) over the total sample, while controlling for pollution and canker/wound status. The Mantel-Haenszel chi-square for each of the two 5 x 3 tables within the high pollution class was significant ($P<0.0001$), but not for the lower pollution class.

Table 4. Impact of the *Armillaria* spp. on sugar maple overall condition (1993-1997), controlling for the global pollutant group and the presence or absence of cankers and/or open wounds on the woody tissues.

Group number	**rRNA-IGS identity**	**Overall tree condition**			**Total number (%[6])**	**Mean Score[7]**
		Healthy number (%[6])	**Affected number (%)**	**Declining/dead number (%)**		
0	No *Armillaria* sp.	385 (35.7)	425 (39.4)	268 (24.9)	1078 (100)	1.89
1	*A. calvescens*	7 (24.2)	13 (44.8)	9 (31.0)	29 (100)	2.07
2	*A. calvescens* & *A. gallica* NA & Eur.	8 (24.3)	11 (33.3)	14 (42.4)	33 (100)	2.18
3	*A. sinapina*	6 (12.8)	19 (40.4)	22 (46.8)	47 (100)	2.34
4	*Armillaria* spp.	16 (16.7)	37 (38.5)	43 (44.8)	96 (100)	2.28

6: For each Armillaria group: percentage of trees in each tree condition category; 7: Mean scores significantly different ($P<0.0001$).

The chances that a tree will be classified as healthy (tree condition 1) rather than affected-declining (tree condition 2 and 3) are 2.3 times higher (95% confidence limits : 1.6-3.4) without Armillaria compared to trees with Armillaria rhizomorphs. Similarly, the chances that a sugar maple will be classified as affected (tree condition 2) rather than very affected, declining or dead (tree condition 3) are 1.7 times higher (95% confidence limits: 1.2-2.4) without Armillaria compared to trees with Armillaria rhizomorphs.

Assessing Armillaria pathogenicity in mature forests is complex, as many species in eastern North America act as secondary pathogens (Gregory et al. 1991; Wargo and Harrington 1991). Results published in observational studies are circumstantial and sometimes contradictory (Dumas 1988; Mallett 1990; Shaw and Loopstra 1988). Cross sectional studies, such as the Portneuf study, yield simultaneous observations of numerous possible causal agents but do not distinguish between predisposing, contributing or inciting stress factors (Manion and Lachance 1992). To establish such a difference, building an experiment in which the environment is manipulated, such as in Bauce and Allen (1992) or Wargo and Houston (1974), is necessary.

Nonetheless, in this study, sugar maples with Armillaria rhizomorphs were significantly less healthy than those without such rhizomorphs penetrating their bark. Moreover, *A. sinapina* was associated with a higher damage level than *A. calvescens*. A further step in assessing Armillaria damage in conjunction with other possible causes would be to model sugar maple health in Portneuf County as related to various amounts of pollutants (F^+, SO_4^{2-}, NO_3^- , H^+), different levels of defoliation, a variety of insect and disease pests, including Armillaria, variable physiological status and the interaction between all these variables, as suggested by Stokes et al. (1995).

CONCLUSION

Genotyping Armillaria rhizomorphs by rRNA-IGS has proven to be a useful tool for the identification of *Armillaria* spp. in the absence of fruiting bodies, provided this genotyping is simultaneously performed on reliably identified specimens as controls.

In Portneuf County, trees without Armillaria rhizomorphs had 2.3 times more chances of being healthy than those with rhizomorphs penetrating their bark, while controlling for the overall pollution level and other woody tissue damage. Almost as many sugar maples were potentially infected by *A. calvescens* than by *A. sinapina* in the Portneuf maple stands. *A. sinapina* was associated with a higher damage level than *A. calvescens*.

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THE EFFECT OF SOIL/ROOT MICROFUNGI ON *ARMILLARIA* RHIZOMORPH FORMATION

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SUMMARY

Ten fungal communities from soil and roots of 3-4-year-old *Betula pendula, Fagus sylvatica, Larix decidua, Prunus serotina* and *Quercus robur* were analysed. The effects of 86 fungal isolates from 56 species from these communities on the formation of rhizomorphs by *Armillaria ostoyae* and *A. gallica* in oak-wood segments *in vitro* were determined. Rhizomorph formation by both *Armillaria* spp. was stimulated by 10 species, of *A. ostoyae* by seven and of *A. gallica* by 14 species. Rhizomorph formation by both *Armillaria* spp. was inhibited by 12 species, of *A. ostoyae* by eight and of *A. gallica* by five species. Inhibitor fungi were isolated more often from soil than from roots. All soil communities were potentially beneficial in having greater proportions of fungi that were inhibitory to rhizomorph formation than were stimulatory. The greatest proportions of inhibitors occurred in soils from *Betula* (90%), *Larix* (78%) and *Prunus* (76%). The only root community with a greater proportion of inhibitors (40%) than stimulants (29%) was on *Prunus*. The least potentially beneficial root communities were on *Quercus* (72% stimulatory) and *Fagus* (70% stimulatory). This suggests that *Betula, Larix* and *Prunus* in a mixed forest may ensure less severe *Armillaria* root rot.

Keywords: *Armillaria,* microfungi, rhizomorphs, roots, soil

INTRODUCTION

In Poland, the main *Armillaria* species infecting conifers, especially *Pinus sylvestris* L., is *A. ostoyae* (Romagn.) Herink. *Armillaria gallica* Marxmüller and Romagn., although it has been generally regarded as a weak pathogen on both coniferous and hardwood hosts, mainly infects weakened broadleaved trees in Poland. *Armillaria gallica* may act in synergy with *A. ostoyae*. Conifer plantations in Poland are damaged by *Armillaria* in the first 5-10 years after planting on sites freshly converted from hardwoods, particularly oaks (*Quercus robur* L.). The common silvicultural practice in these areas is to introduce a mixture of five or six deciduous species, e.g. *Betula, Fagus, Larix, Prunus* and *Quercus.*

The purpose of this study was to determine the composition and function of microfungal communities in the soil and roots of five tree species commonly grown as mixtures, and the effects of the most commonly occurring fungi on the formation of rhizomorphs of *A. ostoyae* and *A. gallica.* An evaluation of the possible effects of the fungal communities on the spread of *Armillaria* in forests is attempted.

MATERIALS AND METHODS

The fungi were isolated from the soil and from10-mm-diam. roots of 3-4-year-old *Betula pendula* Roth., *Fagus sylvatica* L., *Larix decidua* Mill., *Prunus serotina* Ehrh. and *Quercus robur* L. collected in a mixed 10-year-old Scots pine stand in Huta Pusta Forest District (western Poland, 17°10' E, 52°50' N).

Cores of soil were processed separately by the soil dilution plate method (Mańka 1964). Roots were washed under running water and rinsed 10x for 3 min in distilled, sterile water, allowed to dry and, after cutting into 1-mm thick discs, were placed on low-nutrient agar (SNA). Fungi were incubated at 20-22°C for 7-14 days, subsequently identified, and stored at 4°C.

The most frequently occurring fungi were tested for their effects on rhizomorph formation by *A. ostoyae* and *A. gallica*, *in vitro*, in oak-wood sections (Kwasna 2001). The number of rhizomorphs, their length, number of living initials and dry weight were assessed after 6 months in sand and organic substrate at 24°C. Comparisons were made by analysis of variance.

RESULTS

Fungi from ten communities from soil and roots of 3-4-year-old *B. pendula, F. sylvatica, L. decidua, P. serotina* and *Q. robur* were isolated. Sporulating fungi of 24-40 genera were identified in 0.05 mg samples of soil collected beneath the roots of five trees of each species. Fungi of 21-46 genera were identified on 180 root pieces collected from five trees of each species. Frequencies of the most common taxa in the fungal communities are shown in Tables 1 and 2.

Table 1. Frequencies (% of samples) of the most common taxa in fungal communities isolated from soil.

Taxon	***Betula pendula***	***Fagus sylvatica***	***Larix decidua***	***Prunus serotina***	***Quercus robur***
Mucorales	1.2	4.1	2.8	1.4	0.7
Penicillium spp.	94.5	70.7	78.7	83.1	91.5
Penicillium adametzii	70.6	29.4	40.7	52.5	51.1
Penicillium janczewskii	14.9	16.4	21.2	13.5	7.1
Trichoderma spp.	3.5	8.0	5.3	9.1	6.7
Trichoderma viride	1.3	1.7	3.4	6.1	4.1

The effects of 86 fungal isolates from 56 species found in soil and roots on the formation of rhizomorphs of *A. gallica* and *A. ostoyae* in oak segments *in vitro* were determined.

Rhizomorph formation of both *Armillaria* spp. was stimulated by 10 spp.: *Chaetomium cochliodes* Palliser, *Clonostachys* sp., *Cylindrocarpon destructans* (Zinssm.) Scholten, *C. didymum* (Harting) Wollenw., *F. sambucinum* Fuckel, *F. sulphureum* Schlecht., *P. adametzii* Zaleski, (from soil of *Fagus, Larix*) *P. glabrum* (Wehmer) Westl., *P. spinulosum* Thom, *Pseudogymnoascus roseus* Raiłło. Rizomorph formation of *A. ostoyae* was stimulated by seven taxa: *Coniothyrium fuckelii* Sacc., *Monodictis putredinis* (Wallr.) Hughes, *Mortierella gracilis* Linn., *M. microspora* Wolf var. *macrocystis* (Gams) Linn., *Mycelium radicis atrovirens* Melin, dematiaceous hyhomycete, Basidiomycete. Rizomorph formation of *A. gallica* was stimulated by 14 spp.: *Hormiactis candida* Höhn, *M. hygrophila* (from roots of *Fagus*), *M. spinosa* Linn., *Mucor hiemalis* Wehmer, *P. daleae* Zaleski, *P. decumbens* Thom, *P. simplicissimum* (Oudem.) Thom, *P. steckii* Zaleski, *Trichocladium opacum* (Corda) S.Hughes, *Trichoderma atroviride* Karsten, *T. koningii* Oudem., *T. strictipilis* Bissett, *T. viride* Pers., *Varicosporium elodea* Kegel. The darkly pigmented, particularly the black hyphomycetes, e.g. *H. candida* and *M. r. atrovirens* were the strongest stimulants.

Table 2. Frequencies (% of samples) of the most common taxa in fungal communities isolated from roots.

Taxon	***Betula pendula***	***Fagus sylvatica***	***Larix decidua***	***Prunus serotina***	***Quercus robur***
Clonostachys sp.	0	0	0	0	27.1
Cylindrocarpon spp.	5.8	46.5	23.8	25.4	6.4
Mucorales	12.2	12.5	31.4	3.9	3.5
Mycelium radicis atrovirens	55.8	0.7	0	3.9	44.1
Penicillium spp.	3.4	0.3	0.2	13.7	5.2
Trichoderma spp	0	0.7	2.1	23.5	1.1
Trichoderma viride	0	0	2.1	18.6	0
Hyphomycete No. 34	0	0	11.3	0	0

Rhizomorph formation of both *Armillaria* spp. was inhibited by 12 spp.: *Aspergillus kanagawaensis* Nehira, *A. niveus* Blochwitz, *Chrysosporium pannorum* (Link) Hughes, *Penicillium janczewskii* Zaleski, *P. simplicissimum*, *Phoma cava* Schulzer, *Sesquicillium candelabrum* (Bonorden) W.Gams, *Tolypocladium niveum* (Rostrup) Bissett, *Trichoderma harzianum* Rifai, *T. koningii*, *T. viride*, hyphomycete No 34. Rhizomorph formation of *A. ostoyae* was inhibited by eight spp.: *Cladosporium cladosporioides* (Fres.) de Vries, *Cryptosporiopsis radicicola* Kowalski and Bartnik, *M. hiemalis*, *M. spinosa*, *P. adametzii*, *P. daleae*, *Phoma terrestris* Hansen, *Truncatella truncata* (Lev.) Steyaert. Rhizomorph formation of *A. gallica* was inhibited by five spp.: *C. fuckelii*, *Fusarium avenaceum* (Corda) Sacc., *M. hygrophila*, *P. chrysogenum* Thom, *P. soppii* Zaleski. The hyaline fungi, e.g. *S. candelabrum* and hyphomycete No. 34 from *Larix* roots were the strongest inhibitors.

The 'test' fungi had significantly greater influence on rhizomorph production by *A. gallica* than by *A. ostoyae*. In axenic cultures, in the control treatments, *A. gallica* produced rhizomorphs easily while *A. ostoyae* formed only single and short ones. 'Test' fungi affected mostly the number of rhizomorph initials, rhizomorph length and weight, and only rarely the number of rhizomorphs. Some fungi simultaneously inhibited rhizomorph growth by one *Armillaria* sp. and stimulated rhizomorph growth by the other. There were also differences in activity of different isolates belonging to one species. Fungal species inhibiting rhizomorph formation were isolated mostly from the soil of *B. pendula* (27% of all isolates), *F. silvaticus* (27%), *Q. robur* (24%), *L. decidua* (12%) and *P. serotina* (10%). Fungi stimulating rhizomorph formation were isolated mostly from the roots of *F. silvaticus* (34%), *B. pendula* (18%), *Q. robur* (18%), *L. decidua* (18%) and *P. serotina* (12%).

The frequencies of isolates from soils of species that stimulated rhizomorph formation were 27% (*F. sylvaticus*, *Q. robur)*, 17% (*P. serotina*), 10% (*L. decidua*) and 7% (*B. pendula*) and the frequencies that inhibited rhizomorph formation were 90% (*B. pendula)*, 78% (*L. decidua)*, 76% (*P. serotina*), 70% (*Q. robur*) and 60% (*F. sylvaticus*).

The frequencies of isolates from roots of species that stimulated rhizomorph formation were 72% (*Q. robur*), 70% (*F. sylvaticus*), 64% (*B. pendula*), 53% (*L. decidua*) and 29% (*P. serotina*) and the frequencies that inhibited rhizomorph formation were 53% (*L. decidua*), 40% (*P. serotina*), 12% (*B. pendula*), 8% (*F. sylvaticus*) and 7% (*Q. robur*).

DISCUSSION

Shaw and Roth's (1978) statement that 'biological control might be imposed by rhizosphere organisms' prompted the author to take an interest to the effect of soil and root microfungi on growth of *Armillaria*. Considering the mechanism of infection and the behaviour of *Armillaria* in its pathogenic and saprotrophic phases, the useful fungi in biological control will be those which function either by inhibiting or preventing rhizomorph and mycelial development or by limiting the pathogen to the substrate already occupied, partly by actively occupying the substrate. Such fungi are likely to be members of soil/root fungal communities. Of 86 fungal species isolated from the soil and roots of trees, 31 stimulated and 25 inhibited the formation of *Armillaria* rhizomorphs. Although the activity of many of these fungi is recognized, the inability to maintain high populations of organisms inhibitory to the growth of *Armillaria* has been the main factor limiting successful biological control in forests. The application of natural substances may result in only temporary shifts in micorbial populations that lead to short-term biological control. In forests infested by *Armillaria,* a continuous effect is required. It can be achieved only by manipulation of natural populations of organisms by management or by induction of the required populations through the introduction of plants which favour the growth of fungi antagonistic to the pathogen.

The most potentially beneficial fungal communities in soil, i.e. those with the greatest proportions of fungi that were inhibitory, and the smallest proportions that were stimulatory to rhizomorph formation by *A. ostoyae* and *A. gallica*, occurred with *Betula*, *Larix* and *Prunus*. The most potentially beneficial root communities occurred on *Prunus* and *Larix* (on which numbers of inhibitors and stimulants were similar). The most disadvantageous communities occurred on roots of *Quercus*, *Fagus* and *Betula*. This leads to the assumption that a mixture of *Betula*, *Larix* and *Prunus* may ensure less severe *Armillaria* root rot than a mixture composed of *Quercus* and *Fagus*. The strongly inhibitory activity of fungal communities from soils of *Betula*, *Larix* and *Prunus* may be due to the high density of *P. adametzii*, *P. janczewskii* and *Trichoderma* spp. The strongly stimulatory activity of fungal communities from roots of *Fagus* and *Quercus* might be due to the high density of *Clonostachys* sp., *Cylindrocarpon* spp. and *Mycelium radicis atrovirens* Melin.

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EFFECTS OF NUTRIENT AND WATER STRESS ON *ARMILLARIA* DISEASE INCIDENCE IN MARITIME PINE

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SUMMARY

The first objective of this experiment was to evaluate the effects of water conditions and nutrient availability on the incidence of *Armillaria* root disease in maritime pine.

This study was carried out over three years on two-year-old saplings under controlled conditions. Two watering levels were combined with four fertilization levels. *Armillaria* was inoculated twice during the experiment.

The effects of the different watering and fertilization treatments, controlled by measures of mineral status of pine needles, volumic humidity of the compost and predawn leaf water potential of trees, were clearly observed. In the first year of inoculation, water stress caused a significant reduction of tree growth and root biomass. A significant effect of fertilization treatments was observed on root biomass, but only in the third year following fertilization.

The effects of nutrient and water stress on the incidence of *Armillaria* root disease on maritime pine were directly observed and related to root physiology. The total amount of *Armillaria* mortality and infection level were significantly lower in water stressed than in non-stressed trees and in fertilized than in non-fertilized trees. The implications of these results are discussed.

Keywords: *Armillaria* root disease, water stress, fertilization

INTRODUCTION

Armillaria ostoyae is a highly pathogenic species on maritime pines, causing tree death, particularly in the "Landes de Gascogne" forest (South-West France) where intensive forestry practices, using fertilization, are applied. However, many observations in the world suggest that this pathogen can also infect weakened hosts. The major objective of the EU project (which includes this study) is to define the physiological status of maritime pine under different water and nutritional conditions and to forecast the consequences of intensive forestry (fertilization) and global climate change (drought) on pine health.

MATERIAL AND METHODS

The experiment was conducted from March 97 to June 2000. It was carried out on two-year-old saplings under controlled conditions. The 480 maritime pine trees were planted in a greenhouse. The experimental design was set up as a split-plot with main plots for the two watering treatments in each block and four fertilization treatments applied on three sub-plots of 10 trees randomly assigned in the main plots. Analyses of variance (not shown) were carried out for this specific design, using means over sub-plots as variables. Statistical analyses were performed using the SAS package.

Water and fertilization treatments

Treatments were applied in the second and third year of the experiment. Plants were watered using drip irrigation, with one tube per bag. In the second year of the experiment (A), half of the trees were irrigated "nomally", i.e. close to field capacity (NS treatment) and half were water stressed (S treatment). Water stress was applied in two stages: (1) for the entire growing season (March-October), water-stressed plants were provided with half the water given to non-stressed plants, and (2) not watered at all for two summer periods representing a total of three (1999) to five weeks (1998).

The effects of water stress were monitored by measuring the compost volumetric humidity with a TDR probe and the tree predawn leaf water potential with a scholander pressure chamber.

Fertilizer applications were made in spring using a standart fertilizer (Nutricote 14/14/14). Four fertilization treatments were compared: (1) minimal fertilization (F0) i.e. nutrient stress treatment; (2) unbalanced fertilization: low N and K / optimal P (F1) using superphosphate, which is the common forestry practice in the "Landes de Gascogne" forest; (3) sub-optimal fertilization (N1) and (4) optimal fertilization (N2) as the control treatment (unstressed conditions). The effects of fertilization on tree mineral contents were monitored by performing mineral analyses (N, P, K) on needle (reference) and root samples.

Armillaria inoculations

Armillaria was inoculated in June 1998 and June 1999 on separate sub-plots (three per year in each treatment) following the method described by Guillaumin and Lung (1985). Following this inoculation, the occurrence of dead pines was recorded monthly for one year. In addition, at the end of the experiment, assessment of the final aspect of taproots was performed on a scale from 0 (healthy) to 7 (100% invaded by *Armillaria*).

Tree growth parameters

Pine growth in the different treatments was assessed by measuring height and biomass. Seedling height was measured once a year in winter. Sampled plants were oven dried to calculate their total root biomass.

RESULTS

Effects of fertilization and watering treatments on mineral status of maritime pine

The mineral status in the roots at the different times of analyses is given in Figure 1a-b. Nitrogen contents were significantly highest in the optimal complete fertilization (N2 treatment) with a minimum in July 98. No difference was observed between the other treatments. Phosphate contents were significantly highest in the F1 treatment in 1998, and in the N2 treatment in 1999. These results can be explained by the different amounts of phosphorous applied in the F1 and N2 treatments (in the N2 treatment, P levels were 3 times higher than in the F1 treatment in March 1999, and four times higher in March 2000). In general, P contents decreased during the growing season whatever the fertilization treatment due to a dilution effect. Water stress had no significant effect on tree mineral status.

The results of tree predawn water portential measures (Figure 2) showed highly significant differences (P=0.0001) in water status between stressed (S) and unstressed (NS) saplings at the end of the water stress periods (T2, T6, T8). Before water stress (T0) the compost volumic humidity was significantly lower in stressed trees than in unstressed ones. The water stress applied during the two growing seasons was more severe in 1998 than in 1999. Just before the stress period (T0), the compost volumic humidity was significantly lower in 1998 than in 1999 (P=0.002) and the stress period was longer in 1998 (S1) than in 1999 (S2).

Impact of fertilization and water stress on tree growth and root biomass (Figure 3)

The F1 treatment (hyperphosphate fertilization) showed a significant effect on tree height. A significant increase of root biomass was observed in well-fertilized trees (N1 and N2 treatments), in 1999 only. Water stress caused a significant reduction in tree height and in root biomass, in 1998 only.

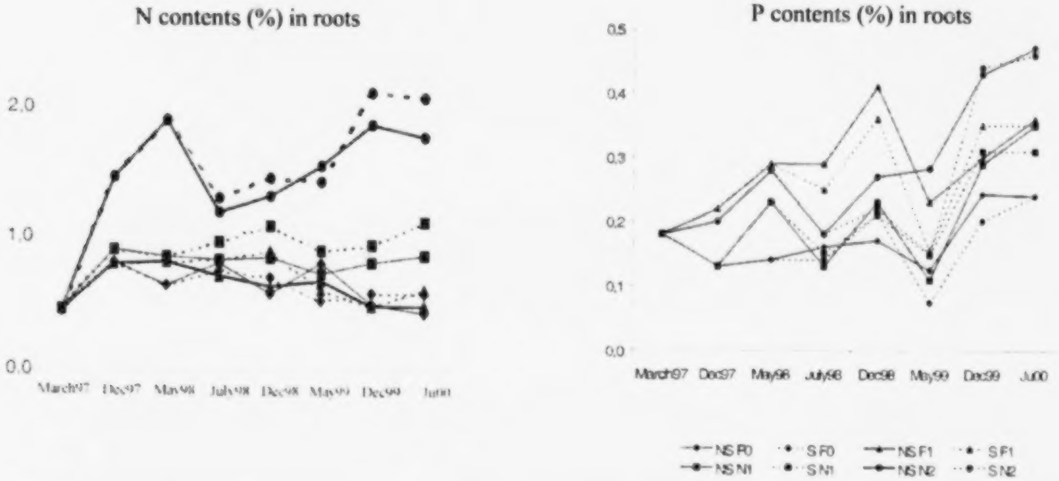

Figure 1. Mineral contents of maritime pines in the different growth treatments throughout the experiment.

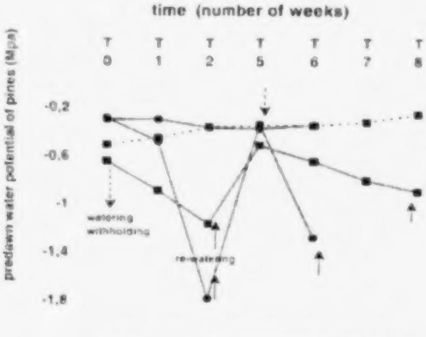

Figure 2. Leaf predawn water potential of maritime pines during the two summer water stress periods: 1 = 1998; 2 = 1999.

Impact of fertilization and water stress on disease performance and impact

In the different fertilization and water treatments, *Armillaria* mortality levels ranged from 29% to 96%. In the first experiment (Figure 4a), the innoculum being still alive one year after inoculation, Armillaria mortality rate (%M) and severity index (results not shown) were significantly higher in unstressed trees than in stressed ones. In the second experiment (Figure 4b), *Armillaria* mortality rate (%M) and severity index (results not shown) were significantly lower in trees with optimal complete fertilization (N2 treatment) than in the other treatments, in the normal watering treatment only.

The effects of the watering treatments in the first experiment only could be explain by more severe water stress applied in 1998 than in 1999. The effects of optimal complete fertilization treatment (N2) in the second experiment only can be corroborated by a significant effect of this fertilization on root biomass only in 1999.

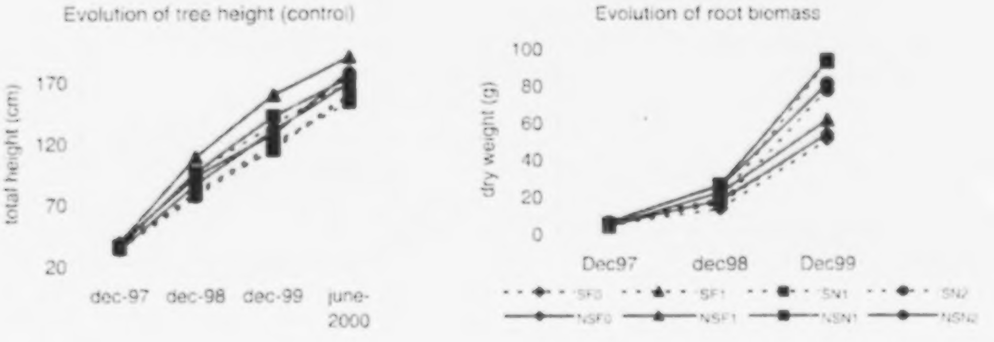

Figure 3. Evolution of tree growth in each treatment throughout the experiment

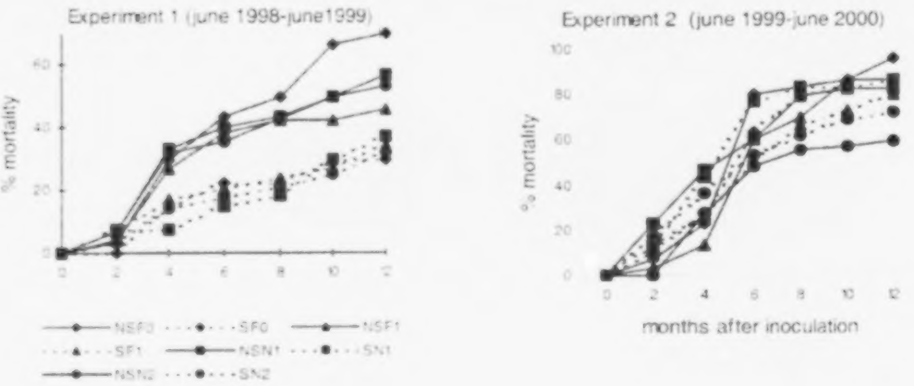

Figure 4a and b. Effects of fertilization and water stress on pine susceptibility to *Armillaria*.

CONCLUSION

Water stress was found to decrease both tree growth and infection of pine trees by *Armillaria* root rot. Nutrient stress decreased tree growth but increased the infection of maritime pine by *Armillaria* root rot. This study confirms that the common fertilization practice of applying hyperphosphate in the "Landes de Gascogne" forest increases the early growth of maritime pines but may not influence their susceptibility to *Armillaria*. The lower susceptibility to *Armillaria* of maritime pines fertilized with nitrogen in the second experiment is consistent with others studies reporting higher *Armillaria* incidence on conifers with decreasing N concentration in soils (Mallet and Maynard 1998, Entry et al. 1991). Inversely, drought is a factor that decreases pine growth and root biomass and it seems to delay the infection process by *Armillaria*. Lower infection levels, on the one hand, in fertilized seedlings, and, on the other hand, on water stress plants cannot be explained by changes in the quantity or quality of terpenes in root tissues (results not shown). The role of water and nutrient supply on the *Armillaria* infection process requires futher investigation.

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AN EXPERIMENTAL STUDY OF THE EFFECTS OF OZONE ON TREE-*ARMILLARIA* INTERACTIONS

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SUMMARY

The effect of ozone on *Armillaria* root diseases was investigated in open top chambers applying a gradient of four environmentally realistic ozone levels. Tree-*Armillaria* interactions studied included *Picea abies-A. ostoyae (*two host provenances-three fungal isolates), *Pinus sylvestris-A. ostoyae*, *Castanea sativa-A. mellea*, and *Robinia pseudoacacia-A. mellea.* Logistic regression analysis showed no significant effect of the ozone treatment on tree mortality for all tree-*Armillaria* interactions. A significant effect of host provenance and fungal isolate on tree mortality was found in the *P. abies-A. ostoyae* interactions. (1) Seedling mortality was higher in the *P. abies* provenance from low-altitude compared to the high-altitude spruce and (2) one *A. ostoyae* isolate killed significantly more seedlings than the other two isolates. Differences in virulence expression among the three *A. ostoyae* isolates were not related to rhizomorph production. The results suggest that the tree-*Armillaria* interactions studied do not respond very sensitive to elevated ozone exposure.

Keywords: *Picea abies*, *Pinus sylvestris*, *Armillaria ostoyae*, root rot, ozone

INTRODUCTION

Ozone is the most important air pollutant during the growing season in many rural areas of industrialized countries. The reactions of woody plant to ozone have been intensively studied for various species (for reviews see Sandermann et al. 1997; Sandermann 1996; Matyssek et al. 1995). Few investigations into the effects of ozone on tree-pathogen interactions have been reported. The majority of plant diseases caused by necrotrophic fungi were found to increase by elevated ozone concentrations (reviewed in Manning and von Tiedermann 1995). It was hypothesized that plants are weakened by ozone stress, thus becoming more susceptible to facultative necrotrophic pathogens.

There are only a few examples where the effect of ozone on root diseases has been studied. Black stain root disease, caused by *Leptographium wagneri* var. *ponderosa* was enhanced in ozone-stressed *Pinus ponderosa* (Fenn et al. 1990). Ozone exposure also increased the disease incidence by *Heterobasidion annosum* root disease in *P. ponderosa* and *P. sylvestris* (James et al. 1980; Bonello et al. 1993). In a field survey, no relation was found between ozone injury and the incidence of Annosus root diseases in *P. strobus* stands (Leininger et al. 1990). Information about the effects of ozone on Armillaria root diseases are lacking. The objective of this research was to investigate the effect of environmentally realistic ozone levels on a number of tree-*Armillaria* interactions.

MATERIAL AND METHODS

The experiment was conducted in 20 open top fumigation chambers described in Landolt et al. (2000). A gradient of four ozone levels was applied in five replications (Table 1). The ambient ozone level is representative for rural areas at low altitude in northern Switzerland while the highest level applied is typical for high altitude sites (1600 - 1800 m a.s.l.) in the Alps. The fumigation treatments were carried out in two growing seasons (1998 and 1999) from the middle of April until the end of September. In October 1999, all plants were removed from the chambers and left under field conditions at ambient ozone concentrations for another year. The third year incubation allowed us to test whether the ozone treatment had a predisposing effect.

Table 1. Ozone concentrations and dose applied in the open-top chambers in 1998 and 1999 (April - September).

Treatment	Mean ozone concentration (ppb)		Ozone dose (AOT40, ppb x h)[1]	
	1998	1999	1998	1999
50% ambient	18.8	16.3	124	21
85% ambient	29.0	26.2	5,118	2,459
100% ambient	34.2	30.9	10,387	6,031
50% ambient + 30ppb ozone	47.2	45.5	17,843	13,291

[1] AOT40, accumulated hourly concentrations over a threshold of 40 ppb during daylight hours (0700 - 1900).

The tree-*Armillaria* interactions studied included *A. ostoyae - Picea abies* (three *A. ostoyae* isolates that exhibited different levels of virulence in previous inoculation trials [Table 2] against two *P. abies* provenances, one from high and one from low-altitude); *A. ostoyae - Pinus sylvestris*; *A. mellea - Castanea sativa,* and *A. mellea - Robinia pseudoacacia.* The two year old tree seedlings or each species were transplanted into polyethylene pots in March 1998. For *P. abies*, five seedlings per pot were used. For all other tree species pots contained only one seedling. An automatic watering system was installed to assure even watering.

Table 2. *Armillaria* isolates used in this study.

Isolate	Year of Isolation	Isolated from	Virulence in previous inoculation tests[1]
A. ostoyae			
3933	1995	Mycelial fans (*Picea abies*)	high
3459	1994	Mycelial fans (*Picea engelmanni*)	moderate
VOR 5-2	1996	Soil rhizomorph from a spruce-fir stand	low
A. mellea			
Tenero 2	1997	Mycelial fans (*Vitis vinifera*)	unknown

[1] Rigling, unpublished results

Inoculations were performed essentially as described by Shaw (1977). In June 1998, each pot was inoculated by inserting an *Armillaria*-colonized hazelnut segment (10 cm long and 2 cm in diameter) without wounding the plants. Autoclaved, non-colonized hazelnut segments were used for control pots. To prepare the inoculum, seven to eight hazelnut segments were placed in a glass jar containing 200 ml of wooden chips (Norway spruce for *A. ostoyae* and hazelnut for *A. mellea*) and 350 ml deionized water. The jars were autoclaved two times for 20 min and inoculated by placing two agar plugs of an *Armillaria* culture onto the wood chips. The jars were incubated at 25°C in the dark for 4 months. Sterile water was added after two months to assure a high moisture content in the jars.

Plants were monthly assessed and mortality recorded. Dead plants were inspected for the presence of mycelial fans at the stem base and the root collars without removing the plants from the pot. Plants showing mycelial fans were considered to be killed by *Armillaria*. Dead plants were left in the pots until the end of the experiment.

At the end of the experiment, the abundance of rhizomorphs in each pot was rated on a four-point scale: 0, none; 1, few; 2, moderately abundant; and 3, abundant. Re-isolates were obtained from at least eight pots of each *Armillaria* isolate. The identity of the re-isolates was confirmed in somatic incompatibility tests.

Mortality data were analyzed using logistic regression for the factors ozone treatment and in the case of spruce, also for the factors host provenance, *Armillaria* isolate, and abundance of rhizomorphs. Chi-square statistic was used to test for independence of abundance of rhizomorphs and the factors ozone treatment, host provenance, and Armillaria isolate. All statistical analyses were performed using DataDesk V. 6.1.1. (DataDescription, Inc. Ithaca, NY, USA)

RESULTS

P. abies-A. ostoyae interactions

There was very little mortality in all interactions in the first growing season after inoculations. Only two low-altitude spruce seedlings were killed by the *A. ostoyae* isolate 3933. Mortality increased during the second growing season. Particularly, low-altitude seedlings were killed by isolate 3933. Mortality further increased in the third year.

The logistic regression analysis was performed for the mortality data obtained two and three years after inoculations. With the exception of the factor host provenance which was not significant in the second year (p = 0.096), all other factors gave a very similar result in both years. The results of the third year are shown in Table 3. Tree mortality was influenced by the factors host provenance and *A. ostoyae* isolate, but not by the factors ozone treatment and abundance of rhizomorphs (Table 3). Seedling mortality was significantly higher in the low-altitude compared to the high-altitude provenance. There was also an effect of the *A. ostoyae* isolate on tree mortality. The *A. ostoyae* isolate 3933 killed significantly more seedlings than the other two fungal isolates. By the end of the experiment, isolate 3933 had killed 27% of the low-altitude and 10% of the high altitude-spruce seedlings. Mortality caused by the *A. ostoyae* isolate 3459 reached only 5% for both provenances. None of seedling died in the pots inoculated with isolate VOR 5-2 and none in the control pots.

There was no significant effect of the ozone treatment on tree mortality, neither after two years (data not shown) nor after three years (Table 3). The lowest mortality in the low-altitude spruce was observed at the highest ozone concentration, however this was not statistically significant.

Table 3. Logistic regression analysis of tree mortality in the *P. abies-A. ostoyae* interactions three years after inoculation.[1]

Source	df	Sums of Squares	Mean Square	F-ratio	P
Ozone treatment	3	3.60	1.20	0.28	0.84
P. abies provenance	1	33.30	33.30	7.89	0.01
A. ostoyae isolate	2	174.24	87.12	20.65	≤ 0.0001
Abundance of rhizomorphs	3	14.71	4.90	1.16	0.32
Error	590	2489.51	4.22		

[1] Data obtained three years after inoculation were analyzed, excluding the control pots without *Armillaria*.

The occurrence of Armillaria rhizomorphs was assessed at the end of the experiment to evaluate whether rhizomorph production correlates with seedling mortality (Table 3) or was influenced by the ozone treatment, spruce provenance, or fungal isolate (Table 4). No relationship between abundance of rhizomorphs and seedling mortality was observed (Table 4). Rhizomorphs were present in almost all of the pots inoculated with the three *A. ostoyae* isolates. Only four out of 150 pots did not contain rhizomorphs. Rhizomorphs were not found in three pots inoculated with isolate 3459 and in one pot inoculated with isolate 3933. Noteworthy, the avirulent isolate VOR5-2 produced rhizomorphs in all pots. However, its rhizomorphs were thinner than the rhizomorphs of the other two isolates. When the abundance of rhizomorphs was considered, isolate VOR5-2 produced significantly fewer rhizomorphs compared to the other two isolates. The abundance of rhizomorphs did not differ between the isolates 3933 and 3459. Pots containing low-altitude spruce contained significant less rhizomorphs than pots with high-altitude spruce. There was no effect of the ozone treatment on rhizomorph production (Table 4).

Table 4. Chi-square statistics for abundance of rhizomorphs in the *P. abies-A. ostoyae* interactions.

Factor	df	Chi-square	P
Ozone treatment	9	12.52	0.18
P. abies provenance	3	5.93	0.11
A. ostoyae isolate	6	35.40	≤ 0.0001

P. sylvestris - A. ostoyae

Two years after inoculation, only one *P. sylvestris* seedling was killed by the *A. ostoyae* isolate 3933. After three years, five plants were dead. Four out of five pines killed by *A. ostoyae* were exposed to low ozone concentrations (50% and 85% ambient), however, logistic regression analysis did not reveal a significant effect of the ozone treatment on pine mortality. Rhizomorphs were found in 19 out of 20 inoculated pots. There was a tendency for a reduced rhizomorph production in pots exposed to high ozone concentrations.

Castanea sativa - A. mellea and *Robinia pseudoacacia - A. mellea*

The *C. sativa-A. mellea* interaction gave inconclusive results. Mortality occurred only in the first two years. A total of seven out of 20 *C. sativa* plants inoculated with *A. mellea* died, however, only four plants showed mycelial fans at the stem base. These four plants were exposed to low ozone concentrations (50% and 85% ambient). There were also three *C. sativa* plants, which died for unknown reason in the control pots. Therefore no further statistical analysis was conducted. Rhizomorphs were found in 17 out of 20 pots inoculated with *A. mellea*. More rhizomorphs were produced at lower ozone concentrations, however, differences were not statistically significant.

None of the *R. pseudoacacia* died during the experiment neither in the inoculated nor in the control pots. Rhizomorphs of *A. mellea* were found in 15 out of 20 inoculated pots.

DISCUSSION AND CONCLUSION

We found no statistical significant effect of the ozone treatment on tree seedling mortality caused by *Armillaria*. In addition, no visible ozone symptoms on the needles of spruce or pine were noted. Our results suggest that the tree-*Armillaria* interactions studied do not respond very sensitive to ozone. Another explanation for the lack of an ozone-effect in our experiment is the relative low ozone exposure applied. The ozone levels chosen were, for northern Switzerland, in a environmentally realistic range. However, compared to other experiments, where ozone-effects on root diseases were observed (Fenn et al. 1990; Bonello et al. 1993), the ozone exposure in our study was mild.

There was a tendency (although not statistically significant) in three tree-*Armillaria* interactions towards less mortality at high ozone concentration. As soil borne pathogens *Armillaria* sp. are probably not directly affected by ozone because of the capability of the soil to absorb ozone (Turner et al. 1973). Therefore, any effect of ozone on root diseases is expected to be plant-mediated. Sandermann (2000) pointed out that ozone acts as effective abiotic elicitor of various plant defense mechanisms eventually resulting in increased pathogen resistance.

One could speculate that elevated ozone concentrations triggered host defense reactions in the whole plant and thereby decreasing Armillaria root disease incidence.

We observed significant less mortality by *A. ostoyae* in the high compared to the low-altitude Norway spruce provenance. Low mortality in high-altitude provenances of spruce was also noted in another inoculation trial (Prospero et al. 2001). The reason for these differences in mortality is not known. It is possible that the low-altitude provenance is more susceptible to *A. ostoyae*. However, an indirect effect of its growth pattern could also play a role. Low-altitude spruce seedlings are growing much faster than high-altitude seedlings which increases the likelihood to contact *Armillaria* and to become infected.

Three *A. ostoyae* isolates, which showed different virulence levels in previous trials, were selected for our experiment to test whether the ozone treatment results in any changes in their virulence expression. The virulence ranking of the three isolates was not affected by the ozone exposure. The virulent isolate 3933 produced the highest mortality in all ozone concentrations. Likewise, the moderately virulent isolate 3459 produced intermediate mortality regardless of the ozone concentrations. The low virulent isolate VOR5-2 did not kill any seedlings, although it produced rhizomorphs in all pots.

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EFFECTS OF STUMP TREATMENTS WITH *PHLEBIOPSIS GIGANTEA* ON MYCODIVERSITY

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In case imported *P. gigantea* preparations are used for stump treatments in countries where genetically similar *P. gigantea* does not occur, the fungus might show unexpected behaviour. We analysed molecular variation within this species and found that *P. gigantea* in Europe is genetically different from that in North-America, where probably two different species of *P. gigantea* exist. Therefore, *P. gigantea* products should not be exported to different continents.

The high amount of a single genotype of *P. gigantea* distributed in forests might reduce the genetic diversity of this species and change the composition of fungal communities colonising stumps. We analysed isolates from single stands and found no dramatic difference in the diversity of *P. gigantea* in one-year-old untreated stumps in stands which had been treated six years before. Thus, the natural spreading of *P. gigantea* into the treated stands seems quite effective in preventing the formation of a genetically monomorphic population. However, the long term effects should be monitored.

We also analysed fungal communities in single stands treated six years before. Most species were not affected by the treatment, but a minority of species seemed to either suffer of benefit from it. However, no differences between the general diversities (as measured by diversity indices) of treated and untreated stumps were observed. Thus, the treatment seemed to have an effect on the species composition but not on the species diversity.

IMPACT OF STAND AND SOIL FACTORS ON THE DISTRIBUTION OF *C. FUSIPES* ROOT ROT IN OAK FORESTS

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The distribution of *C. fusipes* was investigated in three forests of NE France. The presence of basidiomes at the trunk base of oak trees was assessed. The presence and impact of the parasite on the trees were related to stand characteristics such as sylvicultural management, oak species present and tree dbh, and to soil characteristics.

C. fusipes was mainly present in mature forests, on trees over 100 years old, and on *Q. robur*. The main soil factor associated with its distribution was the level of water logging. This parasite was more frequent on soil with traces of water logging appearing deep in the soil. Another important factor was soil texture, *C. fusipes* being especially abundant in soil with a high sand content. By contrast, soil fertility, as measured by soil pH, humus type or the plant community present on the site did not appear to play an important role in the distribution of the parasite. The impact of *C. fusipes* on tree crown appearance was very variable depending on the stands.

Another survey was done in stands selected for their high proportion of infected trees to investigate the relationship between the presence of *C. fusipes* basidiomes at the trunk base and tree crown appearance. Soil texture appeared to be the main factor explaining the difference of impact of *C. fusipes* infection on tree crown appearance.

SPATIAL MOLECULAR ANALYSIS AND MONITORING OF *INONOTUS TOMENTOSUS*

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We have developed molecular tools to study the epidemiology of *Inonotus tomentosus* in spruce stands directly from carpophores or from infected roots. Two DNA primer sets for Polymerase Chain Reaction (PCR) were designed to amplify portions of the large and small subunits of mitochondrial ribosomal DNA. When assayed by Single Strand Conformation Polymorphisme (SSCP) analysis, both genes contained two alleles which resulted in three distinct haplotypes. Specific primers for two nuclear genes, beta-tubuline and actine, were also designed which revealed 27 and 7 alleles, respectively. Over 300 carpophores were sampled in 70-year-old white spruce plantation in western Quebec, and their spatial coordinates were recorded to test hypotheses about clonal and sexual reproduction. The carpophores were genotyped using the four sets of markers. Twenty six genets with distinct DNA profiles were found. Spatial autocorrelation analyses revealed that within class distances of up to 8 m, carpophores were genetically significantly correlated. Comparisons in all other spatial distance classes (i.e. > 8 m) yielded genetic distance values not significantly different from zero. The average genet size was an average of approximately 4 m. The distribution of the genetic polymorphisms is consistent with infections from basidiospores, followed by secondary vegetative spread.

ASSOCIATION OF *INONOTUS TOMENTOSUS* WITH SPRUCE BEETLE ATTACK

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In 1996, spruce beetles (*Dendroctonus rufipennis*) infested the T.W. Daniel Experimental Forest in northern Utah. All trees with spruce beetles were located, and the proportion of infected roots was determined by pit sampling near each stump and at two adjacent points 100 m away. At each sampling point, eight pits approximately 20 cm x 20 cm x 40 cm deep were excavated with a pulaski. All roots >0.5 cm in diameter were examined for root disease symptoms. At seven of the nine spruce beetle infestations, the proportion of roots with symptoms was greater in the area of the infestation than it was 100 m away. There were more roots per sample at the adjacent sampling points than immediately around the beetle attacked trees. *Inonotus tomentosus* was the most abundant pathogen. In this stand spruce beetles attacked first, and continue their attacks in areas of the stand with a greater proportion of symptomatic roots than adjacent, unattacked areas. This evidence suggests that root disease may predispose Engelmann spruce to attack by spruce beetles.

INCIDENCE OF *TOMENTOSUS* ROOT DISEASE RELATIVE TO SPRUCE DENSITY AND SLOPE POSITION IN SOUTH-CENTRAL ALASKA

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Inonotus tomentosus causes Tomentosus root disease in sub-boreal forests of south-central Alaska. Site variables are related to the incidence of *I. tomentosus*.

At three different locations, a total of 371 plots, $50m^2$ each, were examined to measure the incidence of *I. tomentosus* in conjunction with a variety of site variables. These included spruce density, species composition, and slope position (depression, low, mid, upper, flat). The incidence of *I. tomentosus* in plots ranged from 0-100%, and 54% of the plots had no infected trees. No relationship was found between incidence and spruce density (stems/ha or % spruce). There was a significant difference in disease frequency by slope position ($P=0.0013$) with upper slopes having a higher incidence of infected trees. Slope position may be used by forest managers to indicate areas more likely to have a higher incidence of root disease, and can therefore reduce costs of disease surveys.

RED-ROT OF NORWAY SPRUCE (*PICEA ABIES*) IN AUSTRIA – RELATIONS WITH SITE FACTORS BASED ON A SURVEY BY THE AUSTRIAN FORESTRY INVENTORY

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Norway spruce (*Picea abies*), the main tree species of Austrian forests (67%), is suffering from root and butt rots more than from any other biotic damaging agent. Up to 100% of the trees may be devaluated by rot, which can be attributed mainly to *Heterobasidion annosum*.

Within the scope of two surveys (one from 1986 to 1990 and the other from 1992 to 1996), the amount of trees with red-rot has been recorded all over Austria. On the basis of a comparison between the two surveys, tendencies are shown. Relations to numerous site and stand factors such as sea-level, slope, exposition, soil type, stand composition, age class, water balance, etc. are discussed.

THE ROLE OF SOIL MOISTURE CONTENT IN *ARMILLARIA* ROOT DISEASE

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Lodgepole pine and white spruce were grown in peat soil that was kept saturated or at 75%, or 25% of field capacity and inoculated with either *A. ostoyae* or *Armillaria sinapina*. After 12 months, trees were examined for infection, and foliage, stem and root tissues were analysed for nutrient content. Soil moisture had little effect on disease caused by *A. ostoyae* in lodgepole pine and white spruce. *Armillaria ostoyae* inoculum survival was poorest in the saturated soil. Likewise, with *A. sinapina*, disease in white spruce was not affected by soil moisture; however, it was in lodgepole pine. There was progressively more disease in the 75% and saturated treatments. *Armillaria sinapina* inoculum survival was poorest in the 25% treatment and best in the saturated treatment. Potassium concentrations were greater in the foliage and stems of infected trees compared to those of healthy trees.

Control

INTEGRATED CONTROL OF *ARMILLARIA MELLEA* BY *TRICHODERMA HARZIANUM* AND FOSETYL-AL

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SUMMARY

Laboratory, glasshouse, and field experiments have been carried out to integrate a fungal antagonist with a fungicide, fosetyl-Al (Aliette) or fenpropidin (Patrol). An antagonistic isolate of *Trichoderma harzianum* that suppressed Armillaria *mellea in vitro* interacted significantly when application to inoculated strawberry plants was integrated with the systemic fungicides, fosetyl-Al and fenpropidin. Survival was better after treatment with fosetyl-Al than fenpropidin, which was phytotoxic and inhibited the *T. harzianum* inoculant. The antagonist was not adversely affected by fosetyl-Al.

Keywords: fosetyl-Al (Aliette) fungicide, fenpropidin (Patrol), *Trichoderma harzianum*, fungal antagonist, integrated control of *Armillaria mellea*

INTRODUCTION

Apart from soil fumigation after a susceptible crop with carbon disulphide (Bliss 1951), methyl bromide (Vanachter 1979) or chloropicrin (Hagle and Shaw 1991), there is a lack of experimental evidence to support the use of chemical treatments for control of *Armillaria* species (Shaw and Roth 1978), even those like Armillatox, which have been marketed (Redfern 1971). Another similar mixture of cresylic acids (Bray's Emulsion) was also marketed, but again control was not sustained, and there were phytotoxicity problems with both products (West 1994).

A major reason for the failure of both chemical and biological methods is that it is difficult to reach *Armillaria* deep inside wood. Campbell (1934) found that after becoming established in roots, the vegetative mycelium of *Armillaria* develops a protective layer of thick-walled mycelium, the pseudosclerotial envelope, both within the body of the infected wood and the white mycelial fans in the cambium region, similar to the protective layer that also covers the rhizomorphs. Any successful chemical or biological eradicant of the fungus should penetrate into both these resistant structures, yet still maintain its effectiveness.

Using the most active of the newer chemical fungicides evaluated by Turner and Fox (1988), West (1994) found that none could fully eradicate rhizomorphs in soil, even with concentrations as high as 10,000 mg/l, and even a thin layer of bark prevented any of the chemicals from eradicating the underlying mycelium. Despite this, fenpropidin, which exhibited good protectant activity *in vitro*, slowed the rate of mortality of inoculated strawberry, privet and willow, even though it was fungicidal only above 5000 mg/l and fungistatic at lower concentrations. Fosetyl-Al (Aliette) was as active *in vivo* but not *in vitro*. Phosphonic acid, the active ingredient in fosetyl-Al, has been reported by Navarro et al. (1990) to control *Armillaria*.

Although many fungi have also been found to be antagonistic towards *Armillaria* (Leach 1937; Cusson and LaChance 1974; Federov and Bobko 1989; Pearce and Malajczuk 1990; Hagle and Shaw 1991; Riffle 1973; Eghbaltalab et al. 1975), perhaps the most thoroughly studied antagonists are *Trichoderma* species (Hagle and Shaw 1991). In the interactions between *Armillaria* and *Trichoderma*, one critical factor is the tolerance of *Trichoderma* to stress factors that inhibit the growth and metabolism of *Armillaria*.

Several examples of integrated control of *Armillaria* involving *Trichoderma viride* have been reported after fumigation with carbon disulphide (Bliss 1951) and methyl bromide (Munnecke et al. 1973). Munnecke et al. (1981) reported that *Trichoderma* spp. were 1.9 to 3.2 times more resistant to methyl bromide than *A. mellea*, and also suggested that fumigation of soil weakens the defence mechanisms of *Armillaria*.

MATERIALS AND METHODS

The fungicides fosetyl-Al and fenpropidin were applied 40 days before or after the antagonists. Each of the fungicides was applied at 2000 mg/l. Each of the potted strawberry plants was drenched with 500 ml of the fungicide suspensions using a watering can fitted with a sprinkler. The fungicide suspensions were allowed to leach freely from the bottom of the pots. The *T. harzianum* antagonists were grown on 30 g sterile mushroom compost in 500 ml glass jars for 2 weeks before application. Each of the treatments was replicated four times in RCB design. The experiment started on 6 September 1995, and terminated on 25 March 1997.

Along with the above factorial experiment, two other experiments were carried out simultaneously. One of the experiments tested the efficacy of the antagonists alone. Control plants in this experiment were inoculated with *A. mellea* and 30 g sterile mushroom compost, without any antagonist, added around the roots. The other experiment was done to investigate the effect of the fungicides alone on suppression of *Armillaria* root rot. In addition to the dose of the fungicides used in integration with the antagonists (2000 mg/l), two higher doses, 4000 and 8000 mg/l, were tested. Strawberry plants were chosen as a very susceptible host rapidly infected by *A. mellea* (Fox 2000). The strawberry plants were drenched with 500 ml suspension of each dose of the fungicides.

Each of the treatments in these experiments was replicated four times in RCB design. The plants were watered regularly and were sprayed once, on 4 April 1996, with Pentac at 1 ml/l to control red spider mites.

RESULTS

Although no significant differences were found among the antagonists, fungicides or time sequences, the three factors interacted significantly ($P<0.05$). Seventy five percent of the plants treated first with fenpropidin and then, after 40 days, with *T. harzianum*, survived until the end of the experiment which lasted 566 days. None of the plants survived that long when the antagonist and the fungicide were applied in the reverse sequence. *T. harzianum* was more effective when applied before fosetyl-Al and 50% of the plants survived until the end of the experiment when treated in this sequence compared to no plant surviving when the application sequence was reversed. This antagonist interacted differently with fenpropidin as 50% of the plants survived when the fungicide was applied first, compared to 25% when the antagonist was applied first.

The antagonists and fungicides did not differ significantly in terms of mean number of living leaves, but plants treated with the fungicides first had more leaves than those treated with the antagonists first. For the subsequent counts, however, the differences were not significant. The interaction of antagonists with fungicides or time sequences was not significant. The fungicides and time sequences interacted significantly for the number of leaves counted 202 236, and 298 days after inoculation. Plants treated with fenpropidin first (and antagonists later) had significantly ($P<0.05$) more leaves than those treated with antagonists first (and fenpropidin later), while differences in the number of leaves on plants treated with fosetyl-Al in either sequence were not significant. The antagonists, fungicides, and time sequences interacted significantly ($P<0.05$) only 202 days after inoculation. Plants treated with fenpropidin first and *T. harzianum* 40 days later had significantly more leaves than those treated with *T. harzianum* first and fenpropidin 40 days later, while those treated with *T. harzianum* and fosetyl-Al in either sequence had non-significant differences.

The strawberry plants treated with the antagonists alone or left untreated had no significant differences in the number of leaves. Plants treated with the fungicides alone showed significant differences in the number of living leaves counted 236 days after inoculation with *A. mellea*. The number of leaves was significantly higher on plants treated with fosetyl-Al than those treated with fenpropidin. The doses did not differ significantly, and the interaction of the fungicides with the doses was also non-significant.

Neither the effects of the antagonists, fungicides nor timing of the sequences of treatment differed significantly in terms of mean health scores of the strawberry plants. The only significant interaction was between the fungicides and time sequences 202 and 236 days after inoculation. On these dates, the plants treated with fenpropidin first (and antagonists later) had significantly ($P<0.05$) higher health scores than those treated with the antagonists first (and fenpropidin later), while those treated with fosetyl-Al and the antagonists in either sequence did not differ significantly in health scores.

The strawberry plants treated with the antagonists alone or left untreated did not have significant differences in health scores. Plants treated with the fungicides alone showed significant differences only in health scores recorded 236 days after inoculation. Plants treated with fosetyl-Al had significantly higher health scores than those treated with fenpropidin. The doses did not show significant differences and the interaction of fungicides with doses was also non-significant.

DISCUSSION

There was a significant interaction between the antagonists, fungicides, and the time sequence of application. Seventy five percent of the strawberry plants treated first with fenpropidin and then, 40 days later, with *T. harzianum* survived until the end of the experiment lasting 566 days, while none of the plants survived that long when treated with the antagonist first and the fungicide 40 days later. This result shows the sensitivity of the antagonist to the direct application of the fungicide. The antagonist's survival and growth was probably adversely affected when subjected to the direct drenches of the fungicide suspension resulting in greatly reduced activity against the pathogen. When the fungicide was applied 40 days before the antagonist, the residual effect was not large enough to hinder the growth of the antagonist. The frequent watering of the potted plants is not likely to have leached fenpropidin significantly as the fungicide and its metabolites have little or no tendency to leach (Tomlin 1994). West (1994) also found little effects of watering on the leaching of fenpropidin. Fenpropidin binds to soil because of its high affinity for organic matter, a characteristic feature of lipophilic chemicals. The compost used in the pots was rich in organic matter. It was concluded by West (1994) that when fenpropidin had been in the soil for one week or more, it was unavailable for activity against inoculum of *Armillaria*, because of binding to soil. *Armillaria* is generally considered to be more sensitive to chemicals than *Trichoderma* (Bliss 1951; Munnecke et al. 1981). The residual effect of fenpropidin applied 40 days before *Trichoderma* would, therefore, be negligible.

In addition to strong adsorption, fenpropidin is known to be rapidly and extensively degraded in soil (Tomlin 1994) and this process over the 40 day period must also have made it ineffective. Early application of the fungicide also possibly helped in the establishment of *T. harzianum* by reducing the microbial population in the compost that would compete with the antagonist. The survival of the strawberry plants is likely to have resulted from the effect of fenpropidin on Armillaria during the first few days after its application leading to successful control of the weakened pathogen by *T. harzianum* that was applied 40 days later. No plant survived until the end of the experiment when treated with *T. harzianum* alone and only 25% of them survived when treated with fenpropidin alone at the dose rate integrated with the antagonist.

The mechanism by which fenpropidin application, followed by the introduction of *T. harzianum*, suppressed *Armillaria* seems to be similar to the one reported for *T. harzianum* by Bliss (1951) and Munnecke et al. (1981). They suggested that fumigation of soil with carbon disulphide or methyl bromide prevents *Armillaria* from producing antibiotics which makes it susceptible to attack by Trichoderma that survives the fumigation and dominates the treated soil in the absence of other competitive organisms as they are killed by the fumigation. Fosetyl-Al did not work in the same way probably because its impact on *Armillaria*, at the dose rate applied, was not sufficient to prevent it from producing antibiotics and did not eliminate the competing microorganisms to help *T. harzianum*. Findings of the *in vitro* experiments prior to this study support this conclusion.

Integration of *T. harzianum* resulted in the survival of 50% of the strawberry plants when applied before fosetyl-Al or after fenpropidin. Fosetyl-Al suppressed the disease significantly more than fenpropidin. Fifty percent of the strawberry plants treated with fosetyl-Al survived compared to 16.7% of those treated with fenpropidin. Plants treated with the highest dose of fenpropidin (8000 mg/l) showed symptoms of severe phytotoxicity. Though these plants apparently recovered, the treatment was not effective as no plant survived until the end of the experiment. This result indicates that even this high dose failed to eradicate the pathogen inoculum. As discussed earlier, this was probably because of the binding of the fungicide to the soil particles and its rapid and extensive degradation. Fosetyl-Al was found safe and the highest dose of 8000 mg/l did not cause any visible symptoms of phytotoxicity. Instead, this dose was found the most effective, resulting in the survival of 75% of the treated plants.

The fungicides were applied only once in the long duration of the experiment (566 days). Both fenpropidin and fosetyl-Al are reported to be fungistatic rather than fungicidal (West 1994). This would mean that for persistent protection against the disease, the fungicides would have to be applied repeatedly at proper time intervals. The better performance of fosetyl-Al, despite this theoretical limitation, was probably because this chemical can be taken up by the plant roots and stored for prolonged activity. d'Arcy-Lameta and Bompeix (1991) reported that fosetyl-Al was rapidly taken up by tomato roots, and it was postulated that phosphonate, the active compound of this fungicide, can be fixed in the lignified tissues of woody plants and other perennials, allowing its storage and slow release. It seems that for this to happen, the fungicide would have to be applied in heavy concentrations. This should be possible as the fungicide is not phytotoxic.

Previous studies on the integrated control of Armillaria *mellea* have involved affects of phytotoxic fumigants on resident populations of *Trichoderma* in the absence of the living host plant. Integrated control is desirable especially when a living host plant is involved. Most of the fungicides that have been tested previously for the control of *Armillaria* are phytotoxic if applied in high enough concentrations to attempt its eradication and some of them stimulate the growth of the pathogen when applied in low concentrations. To avoid these problems, it was imperative to integrate the fungicides with antagonists at sublethal non-phytotoxic doses. As the antagonist is likely to be adversely affected by the fungicide, the fungicide should be applied first. The antagonist could then be applied after the toxic effects of the fungicide have diminished. This may also help in the establishment of the antagonist, if the soil flora has been killed by the fungicide, thus reducing competition to the introduced antagonist. But if the antagonist is not likely to be adversely affected by the fungicide, then it could be applied before, or simultaneously with, the fungicide. This could have a combined effect on the pathogen resulting, possibly, in its death.

Table 1. Effect of integration of fungicides with antagonists on survival of *Armillaria*-inoculated strawberry plants on median Survival Time (Days)

	A	F	One	Two	One	Two	One	Two
Antag								
Th2	188.5	378.5	221.0	335.0	207.0	188.5	222.5	-
	234.5							
Th23	216.0	238.0	240.5	201.5	-	174.0	198.5	-
	216.0							
Tv3	203.0	171.5	169.0	204.5	165.0	213.5	169.0	180.5
	180.5							
Tham1	198.0	176.0	176.0	187.0	201.5	198.0	147.0	185.0
	185.0							
Co	192.0	218.5	245.0	194.5	253.5	180.5	240.0	218.5
	199.5							
SP	194.5	192.0	180.5	199.5	203.5	194.5	180.5	223.5
	192.0							
	199.5	196.0	191.0	202.0	209.0	188.5	180.5	239.0

***Table 1a.** Effect of integration of fungicides with antagonists on survival of *Armillaria*-inoculated strawberry plants on median Survival Time (Days): Fosetyl-Al (A); Fenpropidin (F)

	Fungicide		**Sequence**		**Fungicide x Sequence**				
	A	**F**	**One**	**Two**	**One**	**Two**	**One**	**Two**	**Antag**
Th2	188.5	378.5	221.0	335.0	207.0	188.5	222.5	-	234.5
Th23	216.0	238.0	240.5	201.5	-	174.0	198.5	-	216.0
Tv3	203.0	171.5	169.0	204.5	165.0	213.5	169.0	180.5	180.5
Tham1	198.0	176.0	176.0	187.0	201.5	198.0	147.0	185.0	185.0
Co	192.0	218.5	245.0	194.5	253.5	180.5	240.0	218.5	199.5
SP	194.5	192.0	180.5	199.5	203.5	194.5	180.5	223.5	192.0
	199.5	196.0	191.0						

Table 1b. Effect of integration of fungicides with antagonists on survival of *Armillaria*-inoculated strawberry plants on median Survival Time (% Survival after 566 days): Fosetyl-AI (A); Fenpropidin (F)

	Fungicide		**Sequence**		**Fungicide x Sequence**				
Antag	**A**	**F**	**One**	**Two**	**One**	**Two**	**One**	**Two**	**Antag**
Th2	12.5	37.5	12.5	37.5	25.0	0.0	0.0	75.0	25.0
Th23	25.0	37.5	37.5	25.0	50.0	0.0	25.0	50.0	31.3
Tv3	0.0	25.0	12.5	12.5	0.0	0.0	25.0	25.0	12.5
Tham1	12.5	0.0	0.0	12.5	0.0	25.0	0.0	0.0	6.3
Co	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SP	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	8.3	16.7	10.4	14.6	12.5	4.2	8.3	25.0	

Table 2. Effect of fungal antagonists on survival of *Armillaria*-inoculated strawberry plants (n=4).

Treatment	**Median Survival Time (Days)**
Control	165.0
Th2	206.5
Th23	212.5
Tv3	154.0
Tham1	192.5
Co	217.5
SP	174.0

Log-Rank Test: X^2-Value (6 d.f.) = 12.11 ($p>X^2 = 0.0596$)

***Table 3.** Effect of fungicides on survival of *Armillaria*-inoculated strawberry plants.

	Median Survival Time (Days)				**% Survival after 566 days**			
Fungicide	**2000 mg/l**	**4000 mg/l**	**8000 mg/l**	**Fungicide**	**2000 mg/l**	**4000 mg/l**	**8000 mg/l**	**Fungicide**
Fosetyl-Al	248.0	-	-	-	25.0	50.0	75.0	50.0
Fenpropidin	199.5	197.0	242.0	197.0	25.0	25.0	0.0	16.7
	231.0	439.5	354.5		25.0	37.5	37.5	

Log-Rank Test: Category	X^2	d.f.	P
Fungicides (n=12)	4.38	1	0.0363
Doses (n=8)	0.57	2	0.7512
Fungicides x Doses (n=4)	6.44	5	0.2656

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* The editors have experimented some problems with the electronic transfer of Tables 1a and 3, and could not answer from the authors before printing to solve these problems. If you need clarification, please contact one of the authors.

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IMPROVING STUMP TREATMENT BY HARVESTING MACHINE

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ABSTRACT

Equipment that enables measurement of the volume of treatment fluid applied to stumps by harvesters and of its collateral waste is described. The equipment was used to demonstrate how the current design of chain-saw bars can be modified to improve coverage and reduce waste, resulting in savings estimated to exceed 30%.

Keywords: Stump treatment, harvester, application rates, wastage

INTRODUCTION

Stump treatment is increasingly used in Europe for the prophylactic control of *Heterobasidion annosum*, and the majority is applied by harvester during felling (Thor and Stenlid 1998; Pratt and Thor 2001). Many systems now use special chain-saw bars for delivery of the protectant, which is pumped on demand from a storage tank to the felling head whence it is discharged onto the stump surface via holes spaced along the length of the chain saw bar . Sophisticated computer control systems may be fitted which determine when the pump is activated, and to an extent this affects the volumes applied. However, treatment inevitably produces waste, either when it is applied in excessive doses, or is sprayed onto the ground surrounding the target stump. The former results in economic loss and is contrary to the spirit of European legislation which seeks to reduce pesticide doses to the minimum (Anon 1994), whilst the latter has environmental implications which may well become unacceptable (Westlund and Nohrstedt 2000). There is clearly a need for a system that can be calibrated for the accurate application of protectants to stumps. There would also be benefit in measuring waste. Direct measurement of the volumes applied to the surface of stumps is not possible using existing technology, and indirect techniques are needed. This paper describes such a method, which allows calculation of the volume applied to stumps by comparing the amount of fluid dispensed at the felling head with the volumes sprayed to waste and captured in a measuring tray.

MATERIALS AND METHODS

The equipment, known as Stump Treatment Calibrator (STC), consists of four instruments which are housed in a portable waterproof (IP64) box, 420 x 305 x 155 mm, gross wt 7 kg, and are powered by an 12v rechargeable battery. The equipment is connected in-line into the delivery pipe of the stump treatment system on a harvester by means of ¼ inch BSP ports and 10 m of standard hydraulic hose. The instruments, mounted in series, consist of a volume/flow meter, pressure meter and two temperature probes in that order. The maximum fluid pressure that the instrument can record is 10 bar. The volume/flow meter displays either the cumulative volume (ml) of liquid discharged, or the current rate of flow (ml/sec) of the liquid in the delivery pipe. The default display shows the cumulative volume and can be reset to zero manually at any time (for example, between stumps). The volume counter continues to update while the flow rate is displayed. The turbine flow sensor is accurate with flows 0.4 – 6.5 litres/min (7 – 108 ml/sec), and is calibrated for use with water or with fluids of similar viscosity. The system is calibrated by comparing the volume delivered from the uncoupled delivery pipe at the harvester head (measured in a measuring cylinder) with the displayed value. Corrections can be made by recalibrating the flow meter. The pressure meter displays the pressure (in bars) in the delivery pipe, and records the maximum and minimum pressures measured. It is reset manually. The current temperature of fluid in the delivery pipe is displayed, and there is an added facility to display remote temperatures at distance (< = 12m) using a thermocouple probe.

To simulate felling and stump treatment, a log of appropriate diameter, 2 – 4 m long, is grasped in the felling head, the lower end resting on a heavy duty steel tray (collecting tray (Fig 1). A disc *circa* 5 – 10 mm thick is cut from the end in a simulated felling cut while the stump-treatment system is activated. The disc is prevented from falling into the collecting tray by a mesh covering the tray. The disc acts as a treated proto-stump. The volume of fluid dispensed is read from the display The waste caught in the tray is poured into a flask for volume measurement. The volume applied to the proto-stump is calculated by subtraction. The fluid incorporates a dye which allows the coverage of each proto-stump to be estimated and described. The spray distribution (i.e. volumes from different parts of the bar when not performing a felling cut) can be measured in a patternator. This is a flat tray constructed out of 2 mm mild steel in the form of a truncated arc of 120°, divided into ten parallel curved channels each 5 cm wide and 2 cm deep. Like the collecting tray, the patternator is placed beneath the felling head and the saw and spray system are activated over it. Measuring bottles fixed at the end of each channel collect fluid sprayed over the tray as the bar describes a simulated felling cut above it. It is assumed that the presence of timber when actually felling could affect the distribution pattern found in this method.

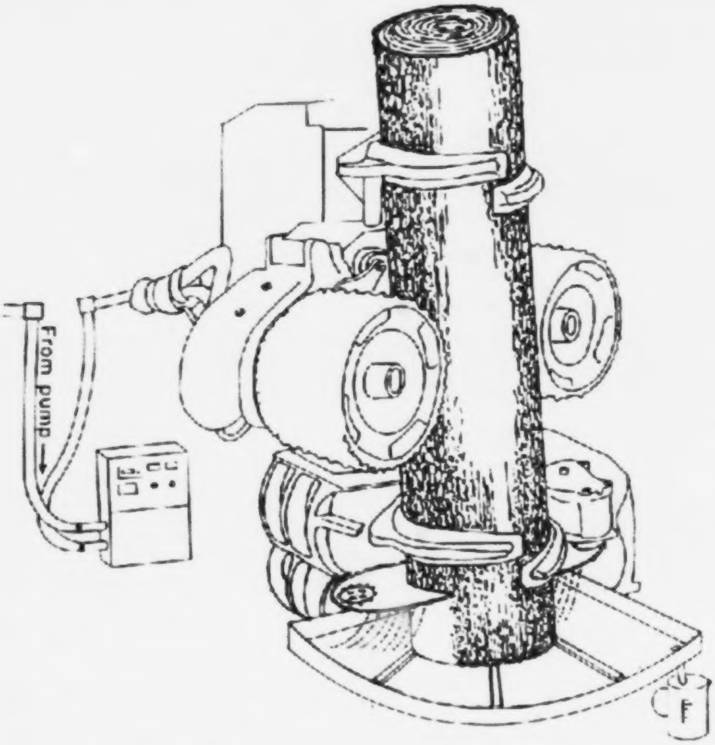

Figure 1. Measuring the distribution of stump treatment fluid applied through a drilled bar. A log, held vertically in the snedding knives of a harvester head, rests on a collecting tray. As the saw cuts through the base of the log (as if simulating the felling of a tree), the treatment fluid that is dispersed to waste either side of the felling cut together with that running off the stump surface is collected and measured in a jug. The flow rate, pressure and temperature is monitored in the STC, which is fitted into the treatment fluid delivery system via a 10 m length of standard hydraulic hose.

RESULTS

1. Standard bars

The distribution of fluid sprayed from a commercially-available standard bar with an evenly-spaced array of 32 holes was measured. The results from three separate trials are summarised in Table 1, in which volumes caught in each pair of adjacent channels are amalgamated for the sake of brevity. The channels and bar holes were numbered sequentially from the sawbox end of the bar (Channel 1and 2, bar holes 1 - 7) to the tip (Channel 10, bar hole 32)

Table 1. Relative distribution of fluid from a standard bar, as measured in the patternator channels.

Patternator channel no.	**1,2**	**3,4**	**5,6**	**7,8**	**9,10**
Bar holes registering on channels	1-7	8-15	16-23	24-31	32
Vol ml (and %) fluid dispensed per channel	201 (30%)	141 (21%)	132 (19%)	106 (16%)	95 (14%)
Area under full sweep of (sq cm)	433	621	811	999	1187
Vol (ml) applied per sq cm	0.46	0.23	0.16	0.11	0.08

As is evident from the table, a third of the fluid was expressed through the 7 holes proximal to the saw box. Because of the configuration of the cutting head, few of these holes would connect with any but the larger stumps, and much of the product would thus be sprayed to waste. In addition, the areas covered by the sweep of the saw bar are much smaller proximal to the sawbox than at the tip, and this provides another cause for uneven distribution of fluid onto a stump along with a higher rate of application than is required (normally 0.1 ml cm^{-2}).

The volumes sprayed to waste using standard bars were measured using the STC and an Oregon 752LUF104 VZ 28167 bar with 42 holes (1.2 mm diameter) set 13 mm apart over a length of 53 cm. Ten proto-stumps each of 35 cm, 21 cm or 12 cm diameter were cut and treated to an acceptable standard (90% minimum coverage of the stump) and the waste was measured in the collecting tray as described above. Waste amounted to 30%, 48% and 55% respectively of the volumes discharged, the greater waste being among the smaller stumps.

2. Improved bars

Observations made in 1999 showed that the holes in a standard bar often extend beyond the dimensions of the stumps being cut. Complete coverage, could be achieved with fewer holes if these were correctly positioned on either side of the centre of the cutting arc. Standard bars were modified by blocking holes, and thus reducing their number (Fig 2). There is clear evidence (Table 2) that in these modified bars fluid is forced laterally out of treatment holes such that adequate coverage can be achieved with significantly reduced doses and less collateral waste.

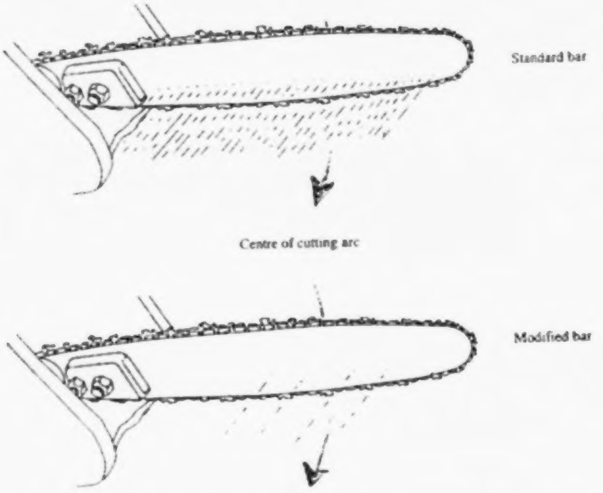

Figure 2. Modifications made to a standard bar involved reducing the number of discharge holes (by blocking unwanted holes), leaving a few placed on either side of the centre of the cutting arc.

Table 2. Comparison between standard and modified bars: mean volumes applied, wastage and stump surface coverage. Means of 10 (or 5) samples per treatment.

Stump diameter cm	Bar type	Number, and length (cm) of array of holes	Waste as a % of discharged volume	Vol. Applied to stump surfaces (litres / sq m)	Coverage Mean %
22	Standard	42, 53cm	48	2.4	100
(N=10)	Modified	3, 13cm	32	1.6	97
44	Standard	42, 53cm	18	1.71	100
(N=5)	Modified	4, 46cm	8	1.07	92

Cutting the number of holes from 42 to 3 or 4 resulted in reduced wastage and in application rates closer to the target of 1 litre/sq m than were achieved with the standard bars. Hole size in the standard bar was 1.2 mm, and in the modified bars 2.1mm. In these trials, fluid was dispensed from the modified bars at a rate of 75 ml/sec. A higher rate (115 ml/sec) was employed for the standard bar to reflect existing operational equipment in Britain.

3. Temperatures and pressures

The temperatures of fluid passing through STC was not elevated significantly above ambient, and did not approach temperatures above 30°C which might have been lethal for biocontrol agents (Thor et al. 1997). Pressures did not exceed 10 bar (1000 kPa), well below a lethal threshold for *Phlebiopsis gigantea* of 2200 kPa (Thor et al. 1997). Careful watch of the meter showed that pressure tends to increase with the flow rate, and with stump diameter. Highest pressure is reached at the end of a cut and may be related to both the time and the length of the cut.

DISCUSSION

This work forms part of a project designed to improve the application of stump treatment and to reduce collateral waste. The STC equipment has, for the first time, allowed parameters to be measured that are important in determining the quality of treatment. The deficiencies of 'standard' bars have been demonstrated, and the benefits from bars tuned both to the geometry of the felling head and stump size have been measured. These findings are contributing towards improvements in bar design. However, there are short-comings with the STC which will need to be addressed. For example, the flow meter is susceptible to producing erroneous results when air permeates the system, and provision of a stop-cock for bleeding out air at the STC is required. Capturing all the waste fluid may require a change in tray design, especially where stumps of large diameter are involved or high pressures are used. We believe the principle could be used to measure the efficiency of any type of treatment systems, such as vertical or horizontal nozzles. There may also be a case for installing part of the equipment within the machine cab, so that the operator can adjust the flow rate and operating pressure to optimise treatment.

APPENDIX

STC components.

Pressure Sensor: IMT Type 3296-075 0 – 10bar, 4-20mA Pressure Transducer	**Digital Thermometers (x2):** CAL Controls Type 3300 K Thermocouple Temperature Control/Indicator
Pressure Meter: Academy Instruments Type A70-24 4-20mA Max-Min Process Meter	**Battery:** Univer Type AB3555EN 4.3Ah 12V NiCad Battery
Turbine Flow Sensor: Roxspur Controls Type FT2-06 0.4 – 6.5 Litres/min, 15Bar max Flow Sensor	**Battery Charger:** Mascot Type 4714 50 – 400mA NiCad Charger
Volume/Flow Meter: Red Lion Controls Type CUB5 Process Counter	**Enclosures (x2):** Himel PLM-32 IP65 GRP Enclosures

ACKNOWLEDGEMENTS

We should like to thank Oregon Cutting Systems® for funding the development of the STC and for taking a positive interest in the work. Colin Saunders (Technical Development, Forest Research) developed the prototype for the system, and we are grateful to him for showing us the way forward.

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IMPACT OF BIOLOGICAL AND CHEMICAL TREATMENTS AGAINST *HETEROBASIDION ANNOSUM* ON NON-TARGET MICRO-ORGANISMS

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SUMMARY

This study describes the effects after one and two years of seven biological and six chemical treatments against *Heterobasidion annosum* on the microfungal communities of *Picea abies* stumps in the western Italian Alps. The treatments influenced, sometimes greatly, the patterns of fungal colonisation of stumps. *Trichoderma harzianum* and *Phlebiopsis gigantea*, among the biological treatments, and sodium tetraborate decahydrate, among the chemical treatments, had the greatest effects on the naturally occurring mycoflora. These effects seem to persist over the time. The impact of the others treatments decreases during the two years.

Keywords: stump mycocenoses, biological control, chemical control, spruce, *Heterobasidion annosum*

INTRODUCTION

Heterobasidion annosum (Fr.) Bref. is a serious pathogen on several important conifers in many parts of the northern Hemisphere. The disease is known to spread via root grafts or spores that infect fresh stumps. Stumps are thus potential reservoirs for *H. annosum* infection and spread and they are targets of biological and chemical treatments (Redfern and Stenlid 1998; Stenlid and Redfern 1998).

Many data exist about the effectiveness of biological (Holdenrieder and Greig 1998) and chemical treatments (Pratt et al. 1998) on stumps, whereas little is known about the environmental hazards of these treatments. Most reports refer to collateral observations on the effects of treatments on target organisms, i.e. fungal pathogens (Rishbeth 1959a, b; Meredith 1960; Butcher 1968; Etheridge 1968; Kallio and Hallaksela 1979), but few detailed information are available about the effects of these treatments on non-target organisms and especially on the fungal population associated with stumps (Rayner 1977a, b; Varese et al. 1999).

Associated with all stages of stump decay is an ancillary succession of nondecay microfungi that usually act as pioneers through their quick colonization of exposed surfaces. They may produce structural and biochemical changes promoting and accelerating natural degradation needed for forest maintenance, and/or oppose decay fungi through direct antagonism and trophic competition (Rayner and Boddy 1988; Dix and Webster 1995; Varese et al. 1999). These fungi should hence be preserved as much as possible and any unwanted shift in the biodiversity of them should be monitored and, if possible, avoided. This is especially important in forest ecosystems, as the integrity of the system is often an important management goal (Kaufmann et al. 1994).

This study describes the effects after one and two years of seven biological treatments and six chemical treatments against *H. annosum* on the microfungal communities of *Picea abies* (L.) Karsten stumps in the northwestern Italian Alps.

MATERIALS AND METHODS

Biological and chemical tests were carried out in two different trials in a Norway spruce stand, situated between 1600 and 2000 m a.s.l. in the Aosta Valley (NW Alps). The forest showed an 80% mean incidence of *H. annosum*.

During the first trial (1994), six biological treatments and one chemical treatment were tested; during the second trial (1997), one biological treatment and five chemical treatments were used. Treatments were applied as indicated in Table 1.

Immediately after felling, 20 healthy stumps were sprayed with each treatment preparation. In the first trial, stump surfaces were protected with an autologous wood disk (cut from the surface of the same stump) to avoid washing away. The controls were 20 untreated stumps covered with an autologous wood disk (C1). Another 20 untreated stumps were left without a wood disk (C2) to evaluate its effects on the naturally occurring mycoflora. In the second trial, the protective wood disk was not used because of its apposition did not influenced or even decreased the effectiveness of treatments (Nicolotti et al. 1999) The number of stumps per treatment was as above.

Table 1. Treatments, application methods and doses

Treatments and Acronyms	Years	Application methods	Concentrations of inocula or active principle in the preparations	Mean doses of inocula or active principle on stump surface
Hypholoma fasciculare (HF)	1994	Wheat mash	4.2×10^{10} CFU/mg	8.4×10^{9} CFU/cm^2
Phanerochaete velutina (PV)	1994	Wheat mash	3.9×10^{19} CFU/mg	9.8×10^{8} CFU/cm^2
Vuilleminia comedens (VC)	1994	Wheat mash	6.7×10^{10} CFU/mg	1.3×10^{10} CFU/cm^2
Trichoderma harzianum (TH)	1994	Conidial and mycelial suspension	9.0×10^{10} CFU/mg	3.1×10^{8} CFU/cm^2
Verticillium bulbillosum (VB)	1994	Conidial and mycelial suspension	6.7×10^{9} CFU/mg	2.3×10^{8} CFU/cm^2
Verticillium bulbillosum (FVB)	1994	Culture filtrate	Concentrated 10 times	3.4×10^{-2} ml/cm^2
Phlebiopsis gigantea (PG)	1997	Rotstop®	H_2O dilution 1 g/l	3.4×10^{-2} ml/cm^2
Sodium tetraborate decahydrate (B)	1997			6.6×10^{-2} g/cm^2
Copper oxychloride (Cu)	1997	Azuram® 40% a.i.	H_2O dilution 10 g/l	3.4×10^{-2} ml/cm^2
Urea 10% (U1)	1997		H_2O dilution 10 g/l	3.4×10^{-2} ml/cm^2
Urea 20% (U2)	1997		H_2O dilution 20 g/l	3.4×10^{-2} ml/cm^2
Urea 30% (U3)	1997		H_2O dilution 30 g/l	3.4×10^{-2} ml/cm^2
Propiconazole (TI)	1994	TILT® 25% emulsion	1% emulsion	3.4×10^{-2} ml/cm^2

The microfungal populations were evaluated one and two years after the treatments by sampling wood slivers from each stump and plating them according to the method described by Varese et al. (1999).

The colonization frequency (F) was computed for each fungus as the number of stumps infected, expressed as a percentage of the total number of stumps examined for each treatment. The colonization density (D) was computed as the number of slivers infected, expressed as a percentage of the total number of slivers plated out for each treatment. Multivariate analysis (CA) of the numerical data were used to assess the effect of each treatment (Syntax V 1993):

RESULTS

From the stumps treated in the first trial, 69 fungal taxa were identified. Of these, 20 were isolated exclusively after one year (first sampling) and 12 exclusively after two years (second sampling). The F values of taxa with a F $\geq$ 60% in one or more treatments are listed in Table 2. The number of fungal taxa is generally significantly lower in the treatments than in C1 after one year, but, after two years, only TH showed significant differences.

Table 2. Colonisation frequency (F) of the fungal taxa during the first trial.

	C1		HF		PV		VC		TH		VB		FBV		TI		C2	
	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs
Absidia cylindrospora	10.0	6.7	47.1*	8.3	63.2*	21.4	15.0	28.6	0	0	10.0	5.6	0	8.3	5.3	25.0	0	10.5
Alternaria alternata	60.0	66.6	5.9*	83.3	15.8*	85.7	0*	92.8	0*	10.0	55.0	83.3	80.0	75.0	26.3*	87.5	25.0*	89.4
Cladosporium cladosporioides	75.0	6.7	5.9*	75.0°	42.1	92.8°	5.0*	57.1°	0*	0	70.0	77.7°	30.0*	58.3°	15.8*	56.2°	0*	63.1°
Cylindrocarpon magnusianum	40.0	13.3	5.9*	25.0	0*	28.5	0*	7.1	10.0*	0°	55.0	11.1	25.0	50.0°	36.8	6.2	75.0	5.3
Epicoccum nigrum	95.0	80.0	29.4*	83.3	57.9	85.7	20.0*	92.8	45.0*	0°	85.0	94.4	100.0	91.6	42.1*	100.0	70.0	100.0
Fusarium tricinctum	15.0	0	0*	0	0*	0	55.0	57.1°	0*	0	0*	0	0*	0	0*	0	0*	0
Gliocladium roseum	5.0	0	0	0	63.2*	71.4°	20.0	28.6	0	0	0	16.7	0	0	0	0	0	0
Graphium an. *Ophiostoma piceae*	0	0	11.8	0	31.6*	0	5.0	0	0	0	60.0*	61.1°	5.0	0	0	0	0	5.3
Mucor hiemalis f. *hiemalis*	80.0	60.0	58.8	100.0	68.4	85.7	40.0	78.6	25.0*	0°	80.0	88.9	70.0	58.3	36.8*	12.5°	50.0	15.8°
Mycelia sterilia dematiacea	30.0	33.3	11.8	8.3°	10.5	28.6°	50	7.1°	0*	0°	30.0	22.2	50.0	0°	57.9	18.7	15	15.8
Penicillium brevicompactum	0	0°	0	33.3°	0	0	90.0*	35.7°	0	0	50.0*	0	0	0	0	0	0	0
Penicillium miczynskii	75.0	46.6	100.0*	83.3°	100.0*	78.5°	95.0	85.7°	0*	0°	40.0*	72.2°	45.0*	16.7°	0*	12.5°	0*	36.8
Penicillium simplicissimum	0	66.6	0	0°	0	57.1	35.0*	0°	0	0°	0	0°	0	8.3°	0	0°	60*	0°
Phoma putaminum.	30.0	60.0	0*	58.3	21.1	71.4	10.0	57.1	10.0	40.0°	70.0*	77.8	15.0	66.6	100.0*	75.0	90.0*	100.0
Trichoderma harzianum	0	0	0	0	0	0	0	0	100.0*	100.0*	0	0	0	0	0	0	0	0
Trichoderma viride	60.0	53.3	70.6	66.7	52.6	35.7°	35.0	78.6	0	0°	25.0*	16.7°	55.0	33.3°	42.1	50.0	60.0	68.4
Verticillium bulbillosum	0	0	0	0	0	0	0	0	0	0	80.0*	44.4°	40.0*	41.6°	0	12.5	0	0
TOTAL ISOLATED TAXA included those with F<60%°	30	25	19*	19	16*	24	18*	19	8*	3°	25	22	26	26	17*	23	14*	22

Most species were significantly enhanced or decreased respect to the control stumps (C1) by one or more treatments and some species were selectively associated with specific treatments. Particularly, *T. harzianum* and *V. bulbillosum*, two of our potential biocontrol agents, were only found, after one year, on the stumps inoculated; after two years, *V. bulbillosum* was also found on stumps treated with propiconazole.

Multivariate analyses illustrated the influence of biological and chemical treatments on the quali-quantitative composition of the microfungal communities of *P. abies* stumps. The treatments that selected the most similar mycocenoses were grouped. One year after the treatments (Fig. 1) TH is separated from the others along the first axis of the scatterplot. Three groups are recognizable along the second axis: the treatments HF-PV-VC, VB and FVB and the C1, TI and C2. The scatterplot relative to the whole data allows the evaluation of the temporal evolution of the effects of the different treatments on the mycoflora (Fig. 2). Two years after, TH treatment is still the most divergent from the control (along the first axis). All the other treatments group together with the control.

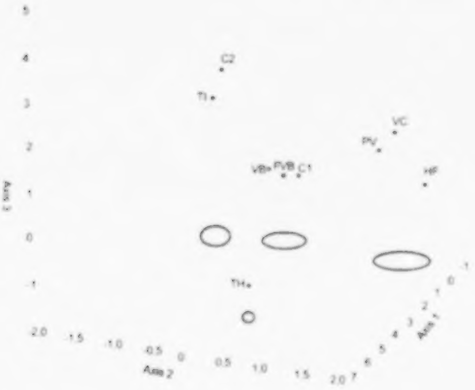

Figure 1. Scatterplot of the correspondence analysis of mycocenoses one year after the treatments (first trial).

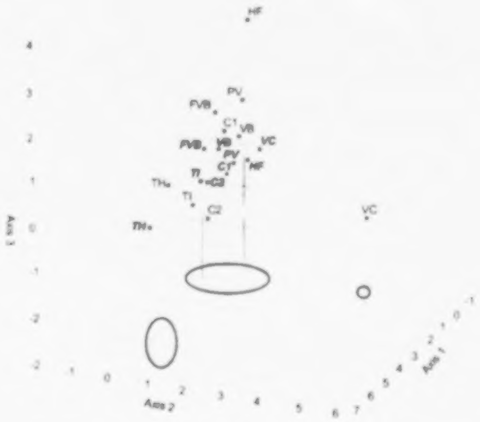

Figure 2. Scatterplot of the correspondence analysis of mycocenoses one and two years (bold and italics) after the treatments (first trial).

From the stumps treated in the second trial, 84 fungal taxa were identified. Of these, 19 were from the first sampling and 27 from the second. The F values of taxa with a F ≥ 60% in one or more treatments are listed in Table 3. The number of fungal taxa is generally lower in the treatments than in C, mainly after one year. However, only B treatment showed significant differences.

Table 3. Colonisation frequency (F) of the fungal taxa during the second trial.

	C		U10		U20		U30		B		CU		PG	
	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yrs	1 yr	2yr
Alternaria alternata	75.0	75.0	30.0	40.0″	50.0	20.0″	10.0	20.0″	100.0•	100.0″	80.0	80.0	38.46	15.4
Cladosporium cladosporioides	25.0	58.3	20.0	10.0″	60.0	40.0	10.0	20.0	0•	0″	20.0	53.3	0•	15.
Cylindrocarpon magnusianum	16.67	25.0	40.0	40.0	0	20.0	90.0•	70.0″	0	0″	0	20.0	0	0″
Epicoccum nigrum	83.3	58.3	60.0	60.0	90.0	50.0	20.0•	0″	0•	0″	33.3•	46.7	15.4•	15.4
Geomyces pannorum	16.7	0	0	0	0	0	0	0	0	7.1	0	0	0	84.5
Mucor hiemalis f. *hiemalis*	91.7	91.7	80.0	70.0	90.0	70.0	80.0	60.0	7.1•	0″	60.0•	0″	84.6	76.
Penicillium purpurogenum	0	0	0	0	0	0	90.0•	0	0	0	0	0	0	0
Penicillium rugulosum	0	0	0	0	30.0″	70.0″	0	20.0	0	0	0	0	7.7	15.
Penicillium verrucosum	16.7	75.0	0	90.0	0	60.0	0	70.0	0	14.3″	13.3	33.3″	0	69.
Penicillum viridicatum	0	0	0	0	0	80.0″	0	40.0″	0	0	0	0	0	0
Phialophora cinerescens	0	0	0	0	0	0	0	0	0	0	66.7•	0	0	0
Phlebiopsis gigantea	0	33.3	0	0″	0	0″	0	20.0	0	0″	0	0″	53.8•	61.
Phoma putaminum.	50.0	83.3	60.0	80.0	80.0•	90.0	30.0	90.0	71.4	42.8″	53.3	20.0″	0•	0″
Ulocladium atrum	16.7	16.7	10.0	10.0	10.0	10.0	0	0	85.7•	85.7″	40.0	40.0	15.4	15.
TOTAL ISOLATED TAXA included those with F<60%[a]	31	24	20	21	20	25	15	22	5•	11″	23	21	23	25

• and ″ values significantly different (Kruscal-Wallis test, p ≤ 0.05) respect to C1 and C after one and two years

Most species were significantly enhanced or decreased respect to control stumps (C) by one or more treatments and some species were selectively associated with specific treatments. It is noteworthy that *P. gigantea* was isolated, after one year, only from stumps treated with this fungus (F = 50%). After two years, that *P. gigantea* was also isolated from the control stumps and from stumps treated with urea 30%.

Correspondence analysis relative to the results one year after the treatments (Fig 3) showed that B is separated from the others along the first axis. Four groups are recognizable along the second axis: the treatments U1, U2 and control (C), the treatment Cu, the treatment U3 and the treatment PG. The scatterplot relative to the whole data (Fig. 4) showed that after two years B treatment is still segregated from the control along the first axis. After two years the treatments with urea (10, 20 and 30%) and with Cu group together with the control, whereas the treatment with PG diverge from it (along the second axis).

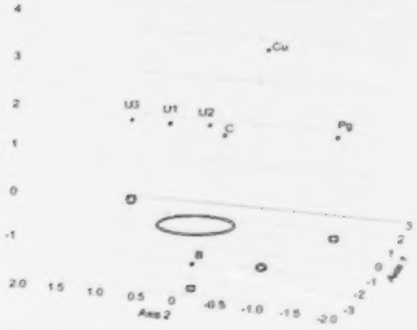

Figure 3. Scatterplot of the correspondence analysis of mycocenoses one year after the treatments (second trial).

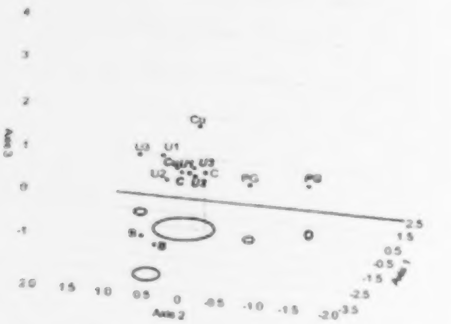

Figure 4. Scatterplot of the correspondence analysis of mycocenoses one and two years (bold and italics) after the treatments (second trial).

DISCUSSION

Many of the isolated species are well known colonizers of stumps of forest trees (Käärik and Rennefelt 1957; Meredith 1959, 1960; Rayner 1977a; Domsch et al. 1980 Mugnai and Capretti 1987; Matta 1996; Nicolotti and Varese 1996).

The qualitative and quantitative predominance of Deuteromycetes has already been reported on Scots pine and black pine stumps (Meredith 1960) and on silver fir stumps (Mugnai and Capretti 1987), and may be partly due to the isolation method we employed, which favors the development of the anamorph rather than the teleomorph.

The presence of Zygomycetes, especially of *Mucor hiemalis* f. *hiemalis* is appreciable. Although mucoraceous fungi are not regularly associated with wood, there are several reports of their occurrence in wood at an advance stage of decomposition (Rayner and Boddy 1988).

The mycocenoesis associated with the control stumps, after one and two years in both trials, are similar to each others, both from a quanti- and qualitative point of view. This would indicate a relative stability of the natural microfungal communities of *P. abies* stumps.

The number of fungal taxa isolated from the treated stumps after one year was always lower than from the control stumps. However, after two years, the number of fungi isolated from treated stumps was generally similar to the one of control stumps. This result suggests that the effects of the different treatments on the biodiversity of the stump fungi decrease over the time, thus enabling the restoration of the natural mycocenosis.

Multivariate analysis illustrated the influence of the biological and chemical treatments on the quali- and quantitative composition of the microfungal community of *Picea abies* stumps.

In the first trial, they also differentiated the two groups of non-treated stumps (C1 and C2).The covered stumps hosted more and differently composed populations, presumably because the disk resulted in greater humidity and acted as a shield against radiation. Thus, the effects of each treatment may be due to both the biological or chemical agent and the presence of the disk. These effects could be either direct or the result of interactions at the community level.

The TH treatment was the most divergent from the control, both after one and two years. It may be supposed that *Trichoderma harzianum*'s high saprotrophic ability and its ecological adaptation to the low forest temperatures encourages its complete stump colonisation and drastically reduced the fungal diversity. Marked reduction of spruce stump biodiversity had been described by Kallio and Hallaksela (1979) in their successful use of *Trichoderma viride* against *Heterobasidion annosum*. *T. harzianum*, however, had no effect on this pathogen in our study (Nicolotti et al. 1999).

The treatments with the three lignivorous Basidiomycetes (HF, PV, VC) showed very similar results; one year after the treatments the related mycocenosis were much more similar to each other than to all the others, however two years after the treatments the differences minimized.

Treatments VB and FVB were the least divergent treatment from the control both after one and two years. Their effects on the fungal community, indeed, were similar to the application of a wood disk only, as in C1. Since they are among the best treatments against *Heterobasidion annosum* (Nicolotti et al. 1999) they can be regarded as a suitable form of biological control.

The mycocenosis associated with the chemical treatment with propiconazole (TI) after one year was different from those of the other treatments and similar to that of C2; after two years the differences minimized. Other chemical compounds have already been shown to selectively and significantly reduce or enhance the incidence of some fungal species (Meredith 1960; Rayner 1977 a,b; Lipponen 1991). In this study, the propiconazole favoured the establishment of *Phoma putaminum* and *Aureobasidium pullulans*, two species whose F values were also high in C2. This finding, referred to a treatment that has been proved to be very effective against *Heterobasidion annosum* (Nicolotti et al., 1999), emphasises the propiconazole's good compatibility with the environment.

The second trial showed that treatments with urea 10% and 20% had the least effects on the stump mycocenosis. These treatments were grouped together with the control both after one and two years. The effects of the treatments with urea 30% and copper were pronounced in the first year however mitigated during the second year, showing that the impact of these treatments decrease over the time.

The treatment with borate had the worst effects on the stump mycocenosis. In both samplings, the number of fungal species isolated from the stumps treated with borate was significant lower than from control stumps. Besides, from a qualitative point of view some species were significantly enhanced and others significantly decreased in their frequencies of colonization. The marked negative impact of this treatment on the microfungal communities seems to persist over the time. This result suggest caution in the evaluation of the ecocompatibility of this treatment since until now borates have shown null or low toxicity for mammalian, plants, fishes and invertebrates (Anonymous 1995, 1996; Pratt 1996).

P. gigantea had a marked effect on the stump mycocenosis as well. This effect is mainly evident on the qualitative composition of the mycoflora. The impact of this treatment on the stump mycocenosis seems to persist over the time and even to increase. It is noteworthy the persistence of this fungus on most of the inoculated stumps and its spread to other treated and untreated ones. The high ability of colonization and persistence of *P. gigantea* on stumps is very important in forest disease management, but it may also represent an environmental hazard. The fungus we used is an exotic strain of an indigenous organism quite rare in Western Italian Alps (Breitenbach and Kräzlin 1986; Bernicchia, personal communication). As underlined by Holdenrieder and Greig (1998), any introduction of exotic living materials present a potential hazard which can lead to an unwanted shift in the biodiversity of the system.

In conclusion, this paper provides evidence that the patterns of fungal colonization of *P. abies* stumps is influenced, sometimes greatly by the treatments against *H. annosum*. Hence, the final choice of a biological or chemical treatment against *H. annosum* should not leave out of consideration the impact of these treatments on non-target organisms inhabiting stumps. The need to prolong the treatments for many years makes particularly important a careful evaluation of the environmental hazard since the possibly negative effects could add up as times goes by.

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GROWTH OF INOCULATED *HETEROBASIDION ANNOSUM* IN ROOTS OF *PICEA ABIES* – EFFECTS OF THINNING AND STUMP TREATMENT WITH *PHLEBIOPSIS GIGANTEA*

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SUMMARY

The spread rate and incidence of *Heterobasidion annosum* (Fr.) Bref. in the roots of Norway spruce (*Picea abies* (L.) Karst.) was studied in three unthinned first rotation Norway spruce stands on former arable land in southwestern Sweden. A known isolate of *H. annosum* was inoculated in 135 trees. One year after the inoculation, 2/3 of the trees were thinned and 1/3 of the trees were left standing. Half of the thinning stumps were treated with spores of *Phlebiopsis gigantea* (Fr.) Jül. and half were left untreated. The length of spread and the incidence of *H. annosum* was investigated three and five years after the inoculation of *H. annosum*. Three years after the inoculation, *H .annosum* had spread significantly longer (50.0 cm) in the roots of the untreated stumps than in the roots of the standing trees (28.8 cm). The length of spread in the roots of the treated stumps was 41.0 cm. Five years after inoculation the length of spread could only be measured in the roots of the standing trees (46.6 cm). The incidence of *H. annosum* was compared at different distances from the inoculation point and was significantly lower for the standing trees than the other treatments.

The study shows that thinning of *P. abies* infected by *H. annosum* will increase the spread rate of *H. annosum* within the root system of decayed trees and the risk for transfer of the disease to adjacent trees.

Keywords: *Picea abies*, *H. annosum*, stump treatment, *Phlebiopsis gigantea*, spread rate, incidence

INTRODUCTION

Root and butt rot causes major economic losses in the Swedish forestry sector. The most important cause of decay is *Heterobasidion annosum* (Fr) Bref. About 15% of all mature Norway spruce trees, *Picea abies* (L.) Karst. in Sweden are infected by root and butt rot (Bengtsson 1975). The main route of infection of *H. annosum* is by airborne basidiospores that establish on freshly cut stumps and wounds, and subsequently spread by growth of mycelia (Rishbeth 1951a,b; Isomäki and Kallio 1974). Since spores are spread during the warm part of the year thinning during this period favours the spread of *H. annosum*. Furthermore, Bendz-Hellgren et al. (1999) found that the growth rate of *H. annosum* in the roots of Norway spruce increased after felling of the infected tree. However, the study by Bendz-Hellgren et al. (1999) was not a true comparison between the growth rate of *H. annosum* in standing trees and stumps since the disease was not introduced in the same way. Consequently forest management without thinning might limit the spread of *H. annosum* in healthy stands as well as in infected stands.

Stump treatment can reduce spore infections on fresh, uninfected spruce stumps by 90-95% (Korhonen et al. 1994, Thor and Stenlid 1997). However, survival of *H. annosum* for 15-60 years in spruce stumps has been reported (Greig and Pratt 1976; Piri 1996). Therefore an infected stump can function as a source of infection for a long time and the effect of stump treatment in infected stands can be questioned. Korhonen et al. (1994) have shown that there is a potential for *Phlebiopsis gigantea* (Fr.) Jül, competing with *H. annosum* in Norway spruce stumps, to reduce the subsequent transfer of *H. annosum* to adjacent trees. However, this statement was based on a small sample. Further investigation was recommended before drawing any practical conclusions.

The aim with this study was to evaluate the growth rate and incidence of *H. annosum*, inoculated in standing *P. abies*, in the root systems of standing trees and stumps and the potential of *P. gigantea* in reducing the growth rate and incidence of *H. annosum* when applied to stumps already infected with *H. annosum*.

MATERIAL AND METHODS

The experiment was established in 1995 in three unthinned first rotation Norway spruce stands on former arable land in southeastern Sweden (56°40' N, 13°10' W). It was designed as a randomised block trial. Each stand consisted of 15 blocks with three trees in each block. The trees were sound and the difference in diameter at breast height between the thickest and the thinnest tree was less than four centimetres. Between each tree in the block at least one growing tree was left. The three trees in each block were inoculated with cutter shavings infected by a known isolate of *H. annosum*.Inoculation was done 10 cm above ground over the main root closest to south, using an increment borer.

In 1996, two trees in each block were randomly selected and felled. The standing tree will be referred to as the T-treatment. The surface on one of the two stumps in each block was manually treated with a suspension of *P. gigantea* oidiospores (Rotstop, 1g/ml) (P-treatment) and the other stump was left untreated (C-treatment).

In the autumn of 1998, seven of the 15 blocks in each stand were randomly selected. The remaining standing tree in each block was felled. In the fresh stumps (T-treatment) and the untreated stumps (C-treatment), all discoloured roots were sampled. The discoloration was measured and discs were taken at 20, 40 and 60 cm from the inoculation point and 10 cm beyond the end of the discoloration. In the stumps treated with *P. gigantea* (P-treatment) discs were cut at 20, 40 and 60 cm from the inoculation point for all main roots. Discoloration was measured and a disc was cut 10 cm beyond the end of the discoloration. All discs were directly transferred into plastic bags and incubated for 10 days. Examination for *H. annosum* in its conidial stage was done using a stereomicroscope. The examination of *P. gigantea* was based on the colour of discoloration and the presence of oidia in mycelia taken from the cut discs.

In the autumn of 2000, seven of the remaining eight blocks on each location were randomly selected. The remaining standing tree on each block was felled and the T-stumps were sampled as in 1998. The C and P-stumps were excavated. Due to the general degradation of these stumps that made it difficult to follow discoloration in the roots, discs were cut 20, 40 and 60 cm from the inoculation point in all main roots. Discs were handled and examined as in 1998. For all stumps in 1998 and 2000, the total number of main roots were counted. The individual of *H. annosum* was tested by using pure cultures made from conidia taken from the sample discs. The pure cultures were grown on agar media (Hagem Agar) together with the known isolate. If no borderline was formed between the two isolates the samples were regarded as being of the same genotype. The individual from 58 roots in 1998 and from 11 roots in 2000 was tested.

The length of spread of *H. annosum* was based on the length of discoloration but adjusted according to the presence of conidia. If the discoloration extended to 60 cm and conidia of *H. annosum* was found at 40 but not at 60 cm the length of discoloration was used, i.e. 60 cm. If the length of spread was 40 cm and conidia was found at 40 and at 60 cm the length of spread was set to 60 cm. A mean of the adjusted values per stump and a mean for each treatment and year were calculated. Due to the general degradation, no numbers for the C- and P-treatments for 2000 are presented. The incidence of *H. annosum* in the roots was calculated as the proportion of main roots with conidia 20, 40 and 60 cm from the inoculation point for each stump. Thereafter the mean of these proportions for each treatment and year was calculated.

The SAS general linear models procedure for split-plot designs (SAS Institute Inc. 1998) was used to perform statistical tests. Differences between individual treatments were evaluated with Tukey's significant difference mean separation test.

RESULTS

In 1998, *H. annosum* had spread longer in the stumps of the C-treatment than in the stumps of the T-treatment ($p<0.05$, Table 1). The P-treatment did not significantly differ from any of the other treatments. Maximum length of spread was 200 cm for the C-treatment, 140 cm for the P-treatment, and 85 cm for the T-treatment. The mean length of spread in 2000, only measured in the T-treatment, was 46.62 cm, and the maximum 80 cm.

The incidence of *H. annosum* in 1998 and in 2000, i.e. the presence of conidia in the discs at 20, 40 and 60 cm distance from the inoculation point, was significantly lower for the T-treatment than for the other treatments, except at 60 cm in 1998, where no difference was found (Table 2). There was no significant difference between the P- and C-treatments.

Both in 1998 and in 2000, 91% of the isolates were of the same genotype as the inoculated isolate. *Phlebiopsis gigantea* was found in 19% of the treated stumps in 1998 and in 83% in 2000. In stumps where *H. annosum* was absent, the presence of *P. gigantea* was the same as in stumps where *H. annosum* was present.

Table 1. Mean spread length of *H. annosum* in roots of *P. abies* based on the length of discoloration in roots adjusted according to the presence of conidia. "C" means untreated stumps, "P" means stumps treated with *P. gigantea*, and "T" is standing trees.

Treatment	**Length of spread (cm)**	
	1998	**2000**
C	50.04 a[1]	
P	40.99 ab	
T	28.76 b	46.62

[1] Figures for length of spread with different letters are significantly different ($p<0.05$).

Table 2. Incidence of *H. annosum* in the roots of *P. abies*, i.e. the proportion of main roots containing *H. annosum* at different distances from the inoculation point. "C" means untreated stumps, "P" means stumps treated with *P. gigantea*, and "T" is standing trees.

	Incidence at different distances from inoculation point								
	20 cm			**40 cm**			**60 cm**		
Treatment	**C**	**P**	**T**	**C**	**P**	**T**	**C**	**P**	**T**
1998	30.4 a[1]	29.5 a	0.8 b	20.8 a	19.8 a	0.0 b	14.4 a	11. 8 a	0.0 a
2000	56.5 a	46.0 a	16.8 b	65.8 a	45.8 a	14.8 b	48.9 a	42.9 a	11.3 b

[1] Figures with different letters are significantly different ($p<0.05$).

DISCUSSION

In this study, the average growth rate of *H. annosum* significantly increased after felling of infected trees. This finding is supported by Bendz-Hellgren et al. (1999). The increased growth of *H. annosum* after felling is probably due to the defence mechanisms of living root wood that is inactivated by felling of the tree. The barrier zones to decay associated with *H. annosum* and *Armillaria mellea* (Vahl. Ex Fr.) Quel. in conifer roots has been described by Tippett and Shigo (1980, 1981).

Corresponding with the study by Bendz-Hellgren et al. (1999), the average growth rate of *H. annosum* in roots of living trees was about 9.5 cm x year^{-1}. Contrarily, the average growth rate of *H. annosum* in stump roots in the study by Bendz-Hellgren et al. (1999) was 25 cm x year^{-1} and about 16.7 cm x year^{-1} for the C-treatment this study. However, in the study by Bendz-Hellgren (1999), *H. annosum* was inoculated into stump roots and in this study the fungus was inoculated in trees that were felled one year later. Therefore, these numbers are not comparable.

The spread length for the C-treatment in 1998 were significantly higher than the spread length for the T-treatment. At the same time the incidence of *H. annosum* identified by its conidial stage was almost zero in the T-treatment. It could therefore be argued that the tree's natural defence in the roots of the T-trees had killed infections of *H. annosum*. This is also supported by Bazzigher (1986) who found a dying off of *H. annosum* in artificially infected Sitka spruce trees over a two-year period. However, in 2000, the spread length had increased and the incidence of *H. annosum* identified by its conidial stage was much higher. Since the T-treatment in 1998 had no infection by *H. annosum* at any distance from the inoculation point except at 20 cm, despite the length of discoloration, and not in 2000, the result can be questioned. It is possible that *H. annosum* was present in the wood without being identified by the presence of its conidial stage in 1998.

H. annosum had on average spread about 9 cm shorter in the P-treatment than in the C-treatment in 1998. The effect of the *P. gigantea* treatment on the growth rate of *H. annosum* was however not significant. Furthermore, the incidence of *H. annosum* was slightly lower, however not significant, for the P-treatment than for the C-treatment in 2000. As there was no difference in incidence but in length of spread in 1998, it could be discussed that *P. gigantea* first slows down the spread rate of *H. annosum* in the root and after that takes over the space initially occupied by *H. annosum*. If the length of discoloration had been possible to measure in 2000, this might have supported this idea. However, Korhonen et al. (1994) have reported growth rates for *P. gigantea* of more than 20 cm in three months. This indicates that *P. gigantea* may have had the potential to quickly compete with and possibly overgrow *H. annosum* in the stumps. However, such a rapid colonization could be questioned since only a few main-roots in the stumps of the P-treatment seemed to be colonised by *P. gigantea* in 1998 and no other significant effects were found.

The incidence of *H. annosum* in roots increased for all treatments between 1998 and 2000. This shows that a higher number of main roots were occupied by *H. annosum* in 2000 than in 1998. As the incidence was estimated for the same distances from the incoculation point in all roots, similar levels of the incidence *H. annosum* could have expected for both sampling years, at least for the C- and T-treatments. However, since the inoculation was carried out on one side of the stump the *H. annosum* infection had to grow across the fibre direction to be able to reach the roots on the opposite side. The growth across the fibre direction may slow down the development of the infection (Rennerfelt 1946).

Due to problems with extensive contamination by bacteria and viruses on agar-cultures, the number of roots tested for the genotype of *H. annosum* became much lower for roots sampled in 2000 than in 1998. Due to the small sample in 2000, it can be argued that other individuals of *H. annosum* than the one first inoculated have been present in the roots. However, as only one of the tested samples was of an unknown genotype in 2000, the influence by other individuals of *H. annosum* should have been small.

CONCLUSION

To conclude, due to an increased spread of the *H. annosum* in roots of infected trees after felling, caution should be shown before taking decision on thinning of Norway spruce stands infected by *H.* annosum. Furthermore, stump treatment with *P. gigantea* may reduce the spread of *H. annosum* in the root systems of stumps already infected by *H. annosum*. However, this was not significant, and therefore additional studies of the influence of *P. gigantea* on the development of *H. annosum* infections are suggested before any further conclusions are drawn.

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EFFECT OF STUMP TREATMENT ON TRANSFER OF *HETEROBASIDION ANNOSUM* ROOT ROT IN NORWAY SPRUCE

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SUMMARY

A stump treatment experiment with Rotstop and urea at 20% and 30% (w/v) was carried out in a first rotation, unthinned Norway spruce stand in Denmark. Six months after inoculation with *H. annosum* using S type conidia, the incidence of infection was 15% (Rotstop), 5% (30% urea) and 3% (20% urea) among the treated stumps and 88% for untreated stumps. The Rotstop treated stumps with infection by *H. annosum* had mostly very small colonies of *H. annosum* and when resampled after another six months, the infection incidence had dropped to 5%. Five years after inoculation, infection had spread to 38% of trees adjacent to untreated stumps but to only 7-14% of trees next to treated stumps. Infection was proved to originate from stumps by somatic compatibility. In a number of cases, the extent of decay and stains could be classed as severe. Based on whether stumps were found to be infected or uninfected in the first assessment (after six months), 43% of trees adjacent to infected stumps (untreated or failed treatment) had rot at the stump surface when examined five years later. In contrast, only 7% of trees adjacent to treated or untreated stumps, that were classed as uninfected in 1995, had rot at the stump surface. It may be concluded that trees next to untreated stumps or to stumps, where treatment has been inadequate, are at greater risk of infection than trees next to stumps, where infection by *H. annosum* has been prevented. An additional finding from this trial was made possible, because the parentage of each tree or stump was known. Part of the observed variation in disease expression among standing trees could thus be accounted for genetically.

Keywords: stump treatment, *Heterobasidion annosum*, rot transfer, *Picea abies*

INTRODUCTION

Since the middle of the 1960's, stump treatment experiments against *Heterobasidion annosum* have been common in Denmark, (Yde-Andersen, 1963, 1966, 1982). Mostly, those experiments were designed to test the efficacy of various protective agents such as creosote, sodium nitrite ($NaNO_3$), urea ($CO(NH_2)_2$) and, recently, Rotstop® (*Phlebiopsis gigantea*). Thus, the experiments mainly consisted of applying stump treatment on half the selected stumps, leaving half untreated, waiting for natural infection, and then returning several months later to take off discs for assessments. Efficacy of an applied agent was considered proven, if none of the treated stumps had colonies of *H. annosum*, identified by the presence of conidiophores after incubation, and the untreated stumps had a significantly higher amount of infection. However, sometimes the experiments were inclusive due to the lack of infection on the untreated stumps. According to the technician in charge of conducting almost all the Danish stump treatment experiments, an infection frequency of 20% on untreated stumps was considered very satisfactory, but seldom achieved when relying on natural infections (M. Egebjerg Petersen, pers. comm.).

Most stump treatment experiments have been carried out in first generation, unthinned conifers, mainly Norway spruce (*Picea abies*) or Scots pine (*Pinus sylvestris*). This reduces the likelihood that *H. annosum* is already present in the stumps, which could influence assessment results. Only rarely have experiments been carried out on sites already infected by *H. annosum*, and the Danish results achieved with urea have not been convincing. Even more rarely have the stump treatment experiments been followed up by later assessments of subsequent transfer into neighbour trees. One very early study (Paludan 1966) illustrated the effect of stump treatment on subsequent spread of *H. annosum*. Results showed that 23% of stumps in the rows treated with creosote were infected by *H. annosum*, whereas 68% of untreated stumps were infected. During the 10 years after stump exposure, 25% of trees next to untreated rows of stumps died due to attack by *H. annosum*. By making wood borings, it was estimated that the fungus was present in up to 42% of trees next to untreated rows. In contrast, only 8% of trees next to rows of treated stumps died, although the fungus was proved present in 4 times the number of trees. Stump treatment thus reduced the impact of *H. annosum*, even though the stump treatment experiment was done with creosote, which in later studies was shown to be even worse than no treatment (Vollbrecht and Jørgensen 1999).

In the last decade, many countries have renewed efforts to find and test stump treatment materials. Sweden and Finland have been especially active in this field. This research led to the introduction of Rotstop®, a formulation of the vegetative spores of *Phlebiopsis gigantea*, and to the recommendation of 30% urea instead of the 20% traditionally used in Denmark. Due to these new findings, a Danish stump treatment experiment was set up in 1995 to test the efficacy of Rotstop and to compare 30% urea with 20% urea. This paper reports the findings concerning the stump treatments and subsequent transfer to living trees.

MATERIALS AND METHODS

The experiment site (F175A) was a first generation Norway spruce planted in 1978 on former agricultural land north of Copenhagen, Denmark. Average rainfall 613 mm per year. The plant material consisted of 3-year-old seedlings originating from controlled crosses between selected mature parent trees. The mating design was factorial with three female clones each mated to a set of 11 other males. The field design was randomized blocks with single-tree plots and 30 blocks. Adjacent to F175A, there were two other plantings (F175B and F209) with plants of other genetic origin. In addition, reference plants from a bulked stand collection (Rye Nørskov) were mixed into all three stands. The stands were mapped, so the genetic origin of each tree was known. On the last two sites, no stump treatments were done and all stumps were inoculated with either an S or a P type of *H. annosum*.

The stump treatment site consisted of 18 rows with 35 trees in each row. Distance between rows was 2 meters. Around the planting a border of two trees was planted to minimise edge effects. Until 1995, no thinnings had been carried out. The heights and diameters had been measured, and Pilodyne readings for wood density had been done for every tree. In 1995 the 20-year-old trees had a mean DBH of 116 mm, varying from 33 to 183 mm.

Stump treatment

In the beginning of June 1995, every third row of trees were cut (row no 2, 5, 8, 11, 14, and 17), leaving 100 cm high stumps. Felled trees were removed from the plot. On June 19, the stump treatments and inoculations were carried out. The treatments were Rotstop, 20% and 30% urea and untreated, and stumps were assigned according to randomised blocks design, with more stumps for the Rotstop treatment and untreated group than the two urea treatments (table 1). Out of a total of 210 stumps, five stumps were rejected, either because the tree had died from suppression or was less than 3 cm in diameter at stump height.

The 100 cm high stumps were cut down to approximately 50 cm with a chain saw, and the treatment applied immediately after cutting. Rotstop was sprayed on the stump surface from a flask containing a suspension made according to the manufacturer's instruction on the bag (1 gr per liter). The urea treatments, 20% (1 kg urea per 4 litres of water) and 30% (1.5 kg urea per 4 litres of water), were applied with a brush. After treatment all stumps (treated and untreated) were inoculated with conidia from an S type isolate of *H. annosum*. The spores were suspended in sterile water and sprayed evenly over the whole surface of every stump. The number of spores pr ml and the exact amount used per stump were not recorded, but inoculum levels were undoubtedly much higher than normal, natural spore infection levels.

After the experiment was set up, the weather turned hot (temp. above 25°C) and dry for several weeks. Five months after inoculation, assessment of stumps began. This took two months, with one row of stumps being assessed every two weeks. The top of the stump was removed and a 2 cm thick disc was cut with a chain saw, put in a paper bag and taken to the lab. Here the discs were wrapped in moist newspaper (outside the paper bags) and incubated for 10 days at 20°C. Each disc was carefully examined for presence of *H. annosum* conidiophores with a stereo microscope. Most of the Rotstop treated discs had turned bright orange in the sapwood, and it was noted when this had not happened on a few discs. Six months after the initial assessment (i.e. one year after inoculation) the treated stumps which had infections, were resampled to check whether *H. annosum* could still be found.

Transfer of *H. annosum*

In June 2000, five years after treatment and inoculation, the remaining rows of trees were cut. Seventeen trees were missing or dead and therefore excluded from assessment and calculations. The stumps were examined for presence of staining or incipient rot. The size of any rot was measured and the maximum extent of the rot column was found by sectioning the stem. Three cases where basal wounds seemed more likely to be the source of infection were noted. With a chain saw stump discs were cut from both the newly felled trees and the untreated stumps from 1995, marked to show positions in the stand (row no and position in row, e.g. 10.57), and taken to the lab in plastic bags. Discs from trees were also marked to show the side facing the previously cut row.

In the lab the position of the rot areas and the relative sizes compared to stump diameter were drawn on circles representing trees. Those stains not resembling typical H. annosum rot were marked with a "?". Isolations were made from discoloured areas by removing small wood samples with an increment hammer and placing them on PDA in petrie dishes. The discs were then incubated in the plastic bags for 10-12 days at 20°C. Presence of *H. annosum* was determined by appearance of conidiophores on incubated discs or by growth of *H. annosum* mycelium from the wood samples. When isolations were successful (some failed due to contamination), the resulting isolates were tested for somatic compatibility against each other. This was done by placing plugs of mycelia 2 cm apart on PDA and observing the mycelial front where the two isolates made contact. If two isolates formed one continuos mat of mycelium with no barrier zone of thickened mycelium or brownish colour, they were considered to be identical.

All the F175A observations from 1995 and 2000 were charted on a map of the whole experiment site (Fig. 1). The map did not illustrate spacing within or between rows or actual size of trees or rot areas. The purpose was to show position of trees and rot areas in relation to treated and untreated stumps, and to depict the rot size relative to each tree. As nearly all untreated stumps were infected, the few uninfected stumps were highlighted by a special mark, whereas treated stumps were usually uninfected and therefore marked only, if they were infected (Fig. 1).

Calculations

From the data collected, several results could be obtained concerning the effects of stump treatment on infection by *H. annosum* and the subsequent transfer into neighbouring trees. The effect of stump treatment against infection of stumps was calculated for both the first assessment after 5-7 months and for Rotstop also 12 months after inoculation. Analysis of variance was used to test the statistical effect and significance of stump treatment in relation to the 1995 results concerning presence of *H. annosum* in stumps.

Rot amount in trees was classified as severe, if the rot height was above 100 cm, or the rot occupied more than half the stump surface. Growth rates of *H. annosum* were calculated by assuming that the distance from inoculation point to stump surface level in neighbour trees was minimum 260 cm. This came from 50 cm stump height in 1995, 200 cm distance between rows, i.e. from stump to nearest tree, and 10 cm stump height in 2000. The average growth rate was calculated by using the actual rot heights of the trees with *H. annosum* transferred from stumps, added with the distance (250 cm) from inoculation point. Finally, the maximum growth rate was calculated from the trees, where the rot height exceeded 100 cm.

Concerning the transfer of *H. annosum* to neighbour trees, rot/stains were excluded if they were considered as not *H. annosum* (12 trees) or thought to originate from wounds rather than stumps (3 trees). These 15 trees were then counted as being without rot. Neighbour trees to the 5 stumps missing in 1995 showed no rot in four cases, but one tree (7.62) next to a missing stump (8.62) had rot towards another stump (8.61) which was untreated. Therefore the two neighbour trees to 8.62 were counted as neighbours to 8.61, but the other neighbours to missing stumps were left out of the calculations. The number of neighbour trees with rot was related to the original treatments of the stumps as well as to whether the stumps were infected or not. Analysis of variance was used to test the statistical effect and significance of stump treatment in relation to the presence of rot in neighbouring trees.

The influence of genetic origin of donor stumps and host trees were tested by analyses of variance of square roots of rot areas on host tree stumps. Square root transformation was applied to counter the skew distribution of this variable. Applying the same statistical method, it was also tested whether transfer of *H. annosum* rot was more likely between stumps and trees with common genetic origin – in this case represented by half-sibs.

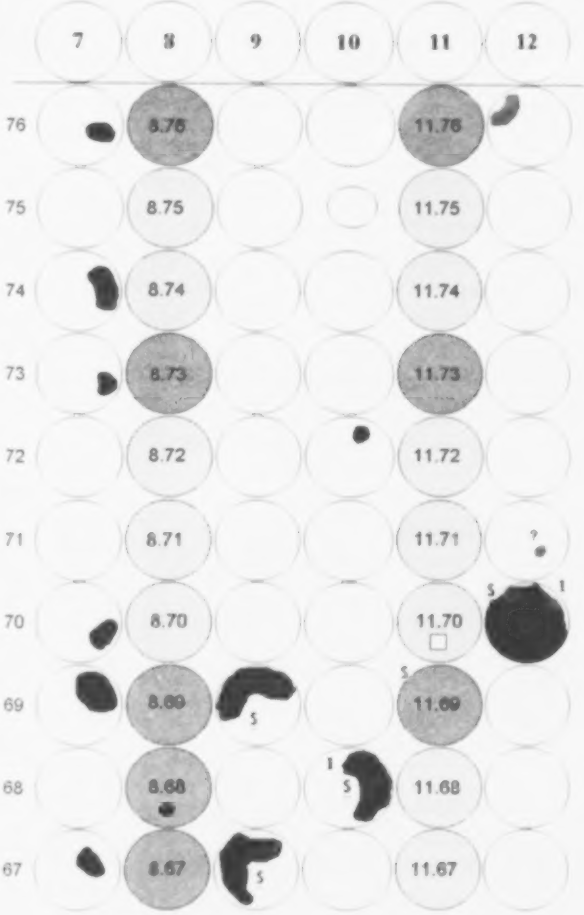

Figure 1. A section from the top of the assessment map with results from F175A. Row numbers are shown in circles above the plot and position within rows to the left. The combination of row and position within rows is shown for stumps from 1995 and can thus be inferred for trees felled in 2000. Dark shaded circles are untreated stumps, of which all shown were infected, except stump 8.68 (black dot mark). Light shaded circles are treated stumps, of which all shown were uninfected, except 11.70 (white square mark). Rot in trees is shown as black stains. The stain with "?" in tree 12.71 was classified as not *H. annosum*, and the *H. annosum* found in 12.76 originated from a wound. The number "1" next to 12.70 and 10.68 shows that rot height was above 100 cm. "S" means that isolates were somatically compatible. The rot in tree 7.70 was towards stump 8.69 rather than 8.70, same for 10.72 towards 11.73. The interpretation could be that rot originated from the two untreated, infected stumps rather than the treated, uninfected stumps.

RESULTS

In the following section "stumps" refer to inoculated stumps of trees cut in 1995 and "trees" refer to trees cut in 2000. "Infection frequency" is used for *H. annosum* infection of stumps in 1995, and "transfer rot frequency" is used for the rot frequency in trees in 2000, when the rot was originating from the stumps inoculated in 2000.

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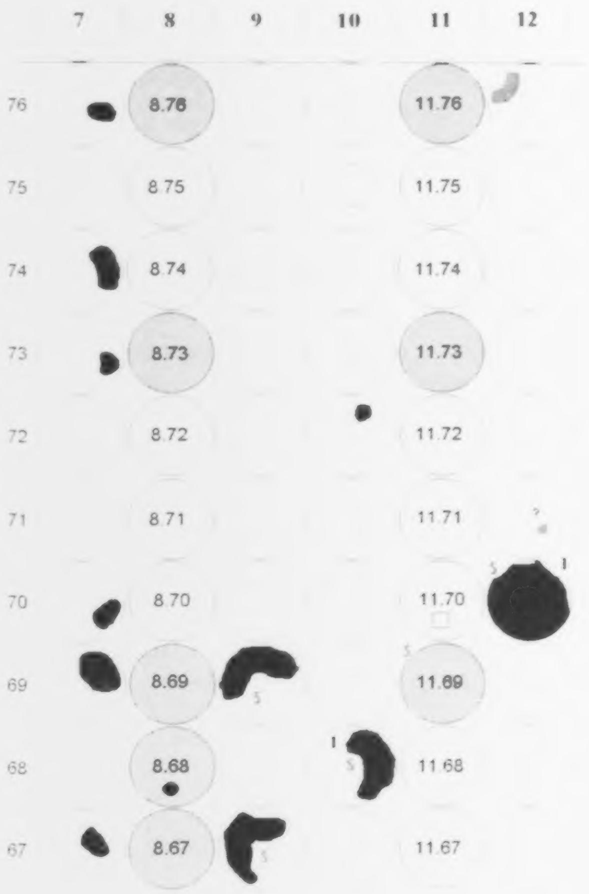

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Stump treatment

Assessment of the stump treatment in 1995 showed that 88% of the untreated stumps were infected by *H. annosum* (table 1). One stump (17.42) counted as uninfected may have had a small colony of *H. annosum*. For the treated stumps, only 2.6% of the stumps treated with 20% urea and 5% of the stumps treated with 30% urea were infected, but 14.5% of the Rotstop treated stumps had infections of *H. annosum*. In most cases, the infected area was rather small, e.g. 1 cm^2 at the edge of the stump or in the heartwood. The rest of the stump was clearly colonized by *P. gigantea* in the sapwood, but not in the small circles of heartwood. This was evident from the orange staining and the strands of *P. gigantea* growing from the edge of the stains. On a few stumps the treatment with Rotstop had clearly failed, as few or no orange stains were present, and large areas were colonized by *H. annosum*. The difference between untreated and treated stumps was significant ($P<0.0001$), but no significant differences could be found between the three treatments.

The second assessment of the treated but infected stumps, six months later, showed that only on three out of nine stumps treated with Rotstop could *H. annosum* still be found. Mostly the stumps were totally overgrown with *P. gigantea*. Infection frequency had thus dropped to 5% or equal to that of 30% urea (Fig. 2). Whether this was a true disappearance of *H. annosum* or just meant that the fungus had grown further down into the stump would not be evident until the subsequent assessment of trees in 2000 (see below).

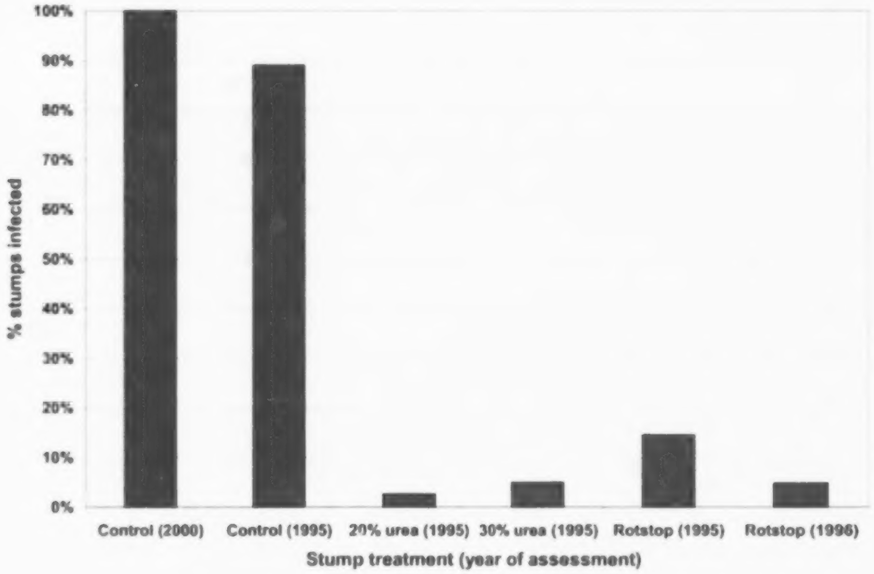

Figure 2. Effect of stump treatment on infection of *H. annosum*. The high infection frequency of the untreated stumps is due to artificial inoculation with *H. annosum* conidia. The second (1996) assessment of the Rotstop treated stumps showed an apparent drop in infection frequency, but the 1995 result was later revealed as more reliable.

Transfer of *H. annosum*

The assessment of trees in 2000 showed that 93 of 403 trees had staining at stump level when felled. If discounting the 15 trees with rot or stains not thought to originate from stumps as well as the neighbours to four missing stumps, in total 78 trees out of 395 showed *H. annosum* rot, a transfer rot frequency of 19.75% (table 1). Nineteen out of the 78 trees had rot heights above 100 cm or more than half the stump surface occupied by rot. Thus 24.4% of the rot could be classified as severe. The minimum growth rate of *H. annosum* to become visible in trees was 260 cm/5 years = 52 cm/year, the average growth rate 62.5 cm/year, and the maximum 76.5 cm/year for the 14 trees with rot heights above 100 cm.

Table 1. Results from assessments in 1995 and 2000. Letters show significant ($P<0.0001$) affiliation of treatments to two significant different groups (untreated and treated stumps). Note two extra trees (next to a missing stump) added as neighbours to an untreated stump, bringing the total to 131, as one neighbour tree was missing. The 25 trees not included are the 17 missing or dead trees from 2000 and the neighbours of the four other missing stumps.

	1995			**2000**	Neighbour trees		
	Stumps inoculated	Stumps infected	Infection frequency	Number of neighbours	All stains and rot	Transfer Rot	Transfer rot frequency
Untreated	65	57	87.7% **a**	129+2	56	50	38.2% **a**
Rotstop	62	9	14.5% **b**	122	21	17	13.9% **b**
Urea 20%	38	1	2.6% **b**	69	10	6	8.7% **b**
Urea 30%	40	2	5.0% **b**	73	6	5	6.8% **b**
	205	69		395	93	78	19.75%
Not included	5	5		25	25	25	
No *H. annosum* seen		169			302	317	
Total	210	210		420	420	420	

A significantly higher number of trees (38.2%) next to untreated stumps had rot compared to trees next to treated stumps (Table 1 and Fig. 3). However, of trees next to Rotstop treated stumps 13.9% had rot, whereas only 6.8 and 8.7% of trees next to urea treated stumps had rot. Urea thus did slightly better than Rotstop, but the differences were not significant. If compared to assessment results in 1995, about 43% of trees next to infected stumps had rot, whereas only around 7% of trees next to uninfected stumps had rot (Fig. 4). The transfer rot frequencies were remarkably similar for infected stumps, whether these were untreated or treatment had failed, even though there were much fewer of the latter. Apparently, some of the Rotstop treated infected stumps on which *H. annosum* could not be found in the 1996 assessment, still were the origin of rot in neighbour trees.

In some cases, the direction of the rot seemed to indicate that it did not originate from the direct neighbour stump, but rather from the stump next to it (Fig. 1). If such a subjective assessment was made, it could be concluded that of the trees with rot 93.6% originated from untreated, infected stumps (79.5%) or from stumps on which treatment had failed (14.1%). In comparison, stumps classified as uninfected in 1995 were only deemed responsible for 6.4% of the rot. Very few stumps on which treatment had been effective could be held responsible for infecting live trees, if the interpretation of rot direction was correct. Two trees had apparently contracted rot from two of the eight untreated, but uninfected stumps. However, one of the stumps was the one (17.42), where *H. annosum* was noted as "probably present". But the results still raise the question whether a few infections were missed in the 1995 assessments.

The analysis of genetic origin of trees showed that the genetic background of stumps did not seem to influence infection success of *H. annosum*. However, the parentage did influence the risk of a living tree becoming attacked. In F175A, significant variation between the three female parents occurred, indicating that offspring of one mother seemed more resistant, and that another female clone gave higher than average susceptibility. Co-ancestry was demonstrated to have a statistical significant effect in trial F175A, in this case half-sib relationship between donor stump and receiving tree. This means that a tree was more likely to be get *H. annosum* rot if the donor stump was related than if it was unrelated. In comparison, none of the neighbouring trials, F175B or F209 showed significant effect of co-ancestry. The findings related to the genetics of trees in all three trials are to be published in more detail later (Wellendorf et al., in prep.).

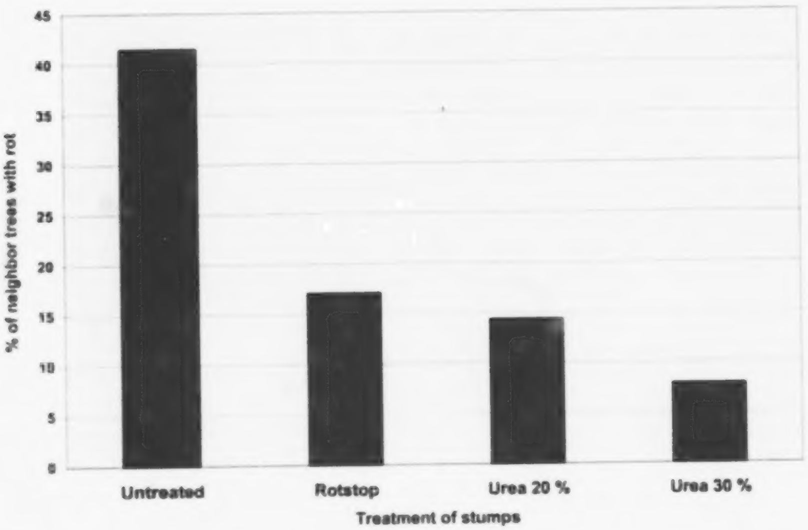

Figure 3. Transfer rot frequency of neighbour trees to stumps with different treatments. The average frequency for treated stumps is 10.6% compared to the 38.2% for untreated stumps. Only the rot stains considered as *H. annosum* coming from stumps are included. The total number of neighbour trees is shown above each column.

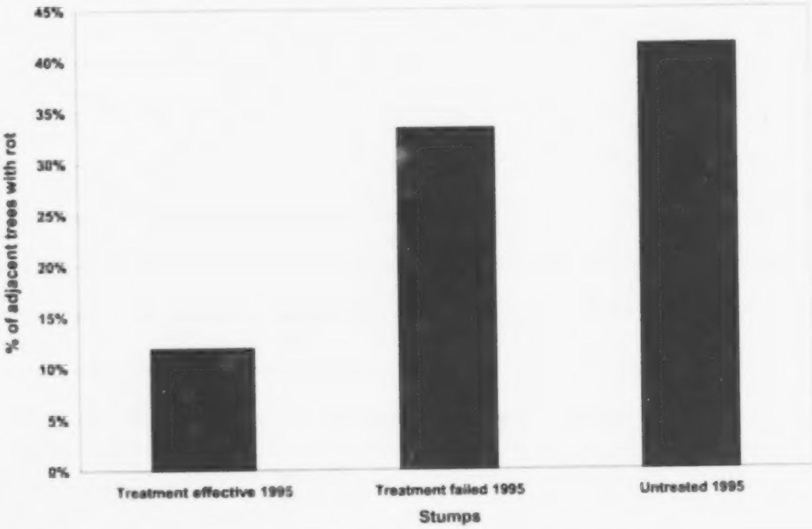

Figure 4. Transfer rot frequency of neighbour trees to infected stumps (middle columns) compared to uninfected stumps. Only the rot stains considered as *H. annosum* from stumps are included. The total number of neighbour trees is shown above each column, revealing that results are most reliable for stumps where treatment had been effective and untreated stumps which had become infected.

DISCUSSION

This experiment gave some interesting results both in relation to the effect of stump treatment and to the subsequent transfer of *H. annosum* to neighbour trees. The implications will be discussed separately.

Stump treatment

The decision to use artificial inoculation in the stump treatment trial meant that the infection risk was much higher than would normally be expected. This was mainly due to the high numbers of conidia, most of which would be dikaryotic, but maybe also due to the water sprayed on the stumps, which wetted the heartwood and might have made it more susceptible to infection. Thus, the slightest failure in stump treatment would probably result in *H. annosum* colonizing the stump. This was evident from the high infection frequency (88%) of the untreated stumps compared to those achieved with natural infection.

The unexpectedly high infection rate (15%) of the Rotstop treated stumps was no doubt also due to the artificial inoculation. In most other trials, *P. gigantea* has give protection equal that of urea or borate, and in this trial, though obvious, the difference was not significantly different. As there was no difference between the effect of 20% and 30% urea treatment, there was no reason to change the traditional recommendation in Denmark. However, the warm weather after treatment may have been an influencing factor, as the apparent breakdown of urea involved in preventing infection by *H. annosum* is strongly dependent on temperature (Johansson et al. 1998). A different result may have been found under cooler conditions. The early 1995 assessment of the treated stumps five months after inoculation turned out to be more correct than the later assessment in 1996 one year after inoculation. This is because even if *H. annosum* could no longer be retrieved from the top of the stumps, it must still have been present, as some neighbour trees subsequently became infected. The few cases where infections may had been missed, serves as a reminder that the method of stump treatment testing is not totally fool proof, but if enough stumps are used, the overall results will be reliable.

Transfer of *H. annosum*

The growth rate of *H. annosum* has previously been estimated as varying between 37 and 86 cm/year, with an average of 52 cm/year (Bendz-Hellgren et al. 1999). However, the same study showed that in average spread rate of discoloration was 36 cm/year, and height of *H. annosum* presence above the discoloration was 60 cm. These averages and the minimum distance of 260 cm from inoculation point to stump height of neighbour tree meant that the expected time for (visible) transfer in the present experiment was 5-7 years. As it turned out, the average spread of discoloration in this study, 62.5 cm/year, corresponds well to the 58 ±8,4 cm/year found on the site with fastest growth of H. annosum in Bendz-Hellgren et al. (1999). Absence of rot at stump height could mean that either the tree has not become infected, or the rot is present in the roots but has not yet reached the butt. No digging of stumps was done to research this problem of underestimating the rot amount. However, the results indicated that most of the *H. annosum* infections had arrived and become visible at stump height. Thus, apart from the average spread rate mentioned above, one fourth of the trees had extensive rot and very few trees had small rot spots just visible at stump height.

Even if some infections were not yet visible, the number of trees infected from stumps was still quite high. In another study, the minimum infection frequency for living trees was calculated as 9.1% ±6.3 % for trees within 2.5 m of thinning stumps (Stenlid 1987). However, the maximum rot frequency was almost 15%, usually found when more than one thinning stump was present within 2 m of trees. The even higher average rot frequency (almost 20%) from the present study no doubt derives from the high stump infection frequency due to artificial inoculation. In addition, this rot frequency was reached within five years compared to eight years in Stenlid (1987) and 6-12 years in Bendz-Hellgren et al. (1999). The decay incidence in the latter study varied from 10 to 66%, but for the site with the fastest growth rate mentioned above, the six years since thinning and decay incidence of 15% are also similar to the present study, although the trees were older (42 compared to 25 years). Thus, the high rot frequency found here as a result of stump infection and subsequent transfer to living trees is not very unusual.

Results also imply that infection of a neighbour tree is as likely to happen from failure of treatment as from no treatment, provided the stump becomes infected (Fig. 4). The risk of natural infection would probably diminish with treatment even if not achieving total covering, because the stump surface area available for infection by *H. annosum* would be smaller than an untreated stump. Even the reduction achieved by stump treatment in this study, i.e. from 38% neighbour trees with rot next to untreated stumps to the average of 11%

neighbour trees with rot next to treated stumps, could be considered satisfactory as the difference was still significant.

However, the most important result might be that 42.4% of neighbours to infected stumps had rot. In contrast, only 7.4% of trees next to stumps assessed as uninfected in 1995 had rot. This implies that lack of (effective) stump treatment in periods with high risks of stump infection will result in considerable amounts of rot in the remaining stand. Even if only 20% of thinning stumps became infected, and 40% of adjacent trees were attacked, this would lead to rot in at least eight trees for every 100 trees felled. The subsequent spread from tree to tree must then be added. More importantly, prevention of stump infection clearly means that standing trees have little risk of being attacked by *H. annosum* (assuming no basal wounds on standing trees).

Shifting the origin of rot for some trees highlighted the importance of stump treatment even more, if the results are correct (i.e. origin assigned correctly). The use of one S clone to inoculate all stumps meant that the specific origin of rot in a tree could not be determined. All that can be proven is that rot did come from inoculated stumps and not from other sources. Further, trees this age and size have quite large lateral roots and could become infected from several directions. However, most of the rot was quite clearly situated towards the neighbouring row of inoculated stumps. In addition, infected stumps had more neighbour trees with rot than uninfected stumps, as 75% of trees with rot were directly next to infected stumps. No tree with *H. annosum* rot was more than one stump away from an infected stump, i.e. there was never a tree with rot in the middle of a stretch of three or more treated, uninfected stumps. Therefore, the conclusion seems valid that most trees with rot came from untreated or treated but infected stumps, but rarely from stumps with effective treatment. Even the three remaining cases could be argued to stem from nearby infected stumps.

The implications of the influence of genetic background are mainly that parents can contribute relative resistance or susceptibility to their offspring. Once the tree is felled, no difference in susceptibility may be found between stumps, indicating that the resistance is related to the defence mechanisms in the living tree, probably the roots. Selection and breeding for resistance to attack by *H. annosum* may therefore be possible. Planting genetically related trees in the same stand may in some cases promote faster rot transfer from stumps to trees, but results were contradictory. However, a stronger effect of co-ancestry might occur, if trees were full-sibs or clones. Furthermore, the impact on tree to tree transfer was not investigated.

CONCLUSION

Both Rotstop and urea were shown to protect stumps from infection by *H. annosum*. Under Danish conditions, 20% urea seems as effective as 30 % urea. Trees next to untreated stumps or to stumps, where treatment has been inadequate, are at risk of infection by *H. annosum*, if this fungus gains entry to the stumps. If stump infection by *H. annosum* is prevented, neighbour trees have almost no risk of becoming infected, provided *H. annosum* is not present in older stumps from earlier generations of conifers. Even if stump treatment is not effective on all stumps, the rot frequency in standing trees will still be diminished significantly, if a high proportion of stumps is protected. Stump treatment can therefore be considered as a useful instrument for prevention *H. annosum* decay in standing trees, even if total protection is not achieved. However, any existing application problems in relation to reaching sufficient coverage and thereby protection should still be addressed. On sites with widespread stump infections by *H. annosum* and optimal conditions for subsequent vegetative spread, rot frequencies in standing trees could reach 20% within 5-10 years after thinning. Up to 25% of this rot could be economically important, due to lateral and vertical extent of discolouration, which necessitates cutting off the lower part (1-2 m) of the timber.

ACKNOWLEDGEMENTS

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OPERATIONAL STUMP TREATMENT AGAINST *HETEROBASIDION ANNOSUM* IN EUROPEAN FORESTRY – CURRENT SITUATION

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SUMMARY

A survey of stump treatment in European forestry was made. Answers were received from nine countries. Stump treatment in thinning was most common, accounting for more than 90% of the area and approximately 65% of the volume harvested. Between them, Great Britain and Poland treat more than 65% of the 200,000 hectares included each year in Europe. Among the three materials available for stump protection, the biological agent *Phlebiopsis gigantea* is used on 64%, urea on 37% and disodium octaborate tetrahydrate on 2% of the area. Issues that need further attention include the technology and methods of application, dissemination of information and training, quality assurance, and the harmonisation of legislation on pesticides within the EU.

INTRODUCTION

Root and butt rot caused by the fungus *Heterobasidion annosum* is damaging in stands and plantations of conifers in Europe: estimated losses caused by the disease amount to 790 million euro for Europe alone (Woodward et al. 1998). The fruiting bodies of the fungus produce spores in large amounts. Following their germination on freshly-cut surfaces of conifer stumps, the fungus may grow down into woody root tissue and spread via root contacts to healthy trees standing nearby. Both thinning stumps (Rishbeth 1949) and clear-fell stumps (Rönnberg and Jörgensen 2000) are at risk from air-borne infection. The disease appears as heartwood decay in spruce, fir or larch, or the death of pine. As the fungus may remain alive in stumps for many years, these can act as a source of infection for young trees in the subsequent rotation.

The disease can be avoided by treating fresh stumps with chemical or biological control agents (Rishbeth 1959, 1963) to prevent the spores from germinating on the stump surface. When introduced in Great Britain during the 1960s, stump treatment was carried out manually. As a consequence of mechanization in forestry, however, much treatment is now automatic, applied during logging with single-grip harvesters.

The control agent used must meet a number of criteria, e.g. be efficacious in a wide range of conditions, cheap, non-toxic, bio-degradable and approved for use. Three materials that in principal meet these criteria are available in Europe; urea, disodium octaborate tetrahydrate (DOT) and the fungus *Phlebiopsis gigantea*. Urea and DOT are chemical compounds, whereas *P. gigantea* is a biological control agent. Each of these materials has its advantages and disadvantages, and the choice of which to use is often dictated by local factors. Because the market for stump treatment materials is comparably small and the costs of registration and product development are very high, it is unlikely that any new control agents will be developed and registered in the near future. The systems needed to be installed or fitted to harvesters include a storage tank for liquid, pipework from the tank to the harvester head, a spaying device and a control unit (preferably integrated with the harvester's control system). The spraying device may consist of a special bar or a spray nozzle. Although it has been demonstrated that effective application by harvesters is possible (Thor and Stenlid 1998), there is still ample scope for improvement, both as regards technical and organisational issues (Pratt and Thor 2001): for example, co-operative research on a European scale was suggested, where besides of the researchers, policy-makers have an important role to play. In order to get a clearer view of the areas subjected to stump treatment, the materials used and current trends in the operation as it is undertaken in Europe a survey was carried out.

MATERIAL AND METHODS

A questionnaire was sent out to chosen representatives from Denmark, Finland, France, Germany, Great Britain, Ireland, Norway, Poland and Sweden. Answers were received from all countries. The country representatives were:

Kari Korhonen, Metla, Finland
John Lyons, Coillte, Ireland
Berthold Metzler, Forstliche Versuchs- und Forschungsanstalt, Germany
Jim Pratt, Forest Research, Scotland
Zbig Sierota, Forest Research Institute in Warzaw, Poland
Halvor Solheim, NISK, Norway
Alain Soutrenon, Cemagref, France
Iben Thomsen, The Research Centre for Forest and Landscape, Denmark
Magnus Thor, SkogForsk, Sweden

The data from each country were analysed and compiled in Microsoft Excel. If any answer was given as an interval, the mean value in the interval was registered.

RESULTS

Great Britain and Poland together accounted for about 65% of the total area (210,000 hectares) treated annually (Table 1). Most treatments were carried out in thinning, which accounted for 90% of the reported area treated. Stumps of *Picea spp*, mainly *P. abies* and *P. sitchensis*, were the predominant tree species, although in Poland stump treatment was only carried out in stands of *Pinus silvestris*. Another pine species treated was *Pinus nigra*. 'Other Conifers' in the table refer to *Picea*, *Pinus* and *Larix* species which were regarded as minor by the country representative. Other conifers included, for example, *Abies alba*, *Abies grandis*, *Pinus pinaster*, and *Pseudotsuga menziesii*. In Germany, stump treatment was only carried out on experimental plots and not in practical forestry.

Table 1. Area treated for all countries in the survey, and the distribution of treatment of various tree species (the distribution is calculated from estimated percentage values, why the figures may appear more exact than they actually are).

Country	**Area treated (ha · yr^{-1})**		**%**	**Tree species (ha · yr^{-1})**			
	thinning	**clearfell**	**of area**	***Picea spp***	***Pinus spp***	***Larix spp***	**Other conifers**
Denmark	5 000	500	3%	3 575	0	0	1 925
Finland	9 000	3 000	6%	10 200	1 800	0	0
France	300	0	<1%	207	33	0	60
Germany	Only on	experimental	Plots				
Great Britain	60 000	9 000	33%	53 820	6 900	6 900	1 380
Ireland	9 950	8 500	9%	13 653	3 505	553	738
Norway	400	0	<1%	400	0	0	0
Poland	70 000	10	33%	0	70 010	0	0
Sweden	35 000	50	17%	35 050	0	0	0
Sum	*189 650*	*21 060*	*100%*	*116 525*	*82 248*	*7 453*	*4 103*

Generally, the trends concerning treated area were reported to be increasing or stable. However, from Great Britain it was estimated that the treated area in thinning should decrease over the next five years until a policy of continuous cover is introduced. Finland, France and Sweden estimated stump treatment to increase in both thinning and final felling, whereas Poland estimated an increase only in thinning. From the rest of the countries the situation were reported stable.

The biological control agent *P. gigantea* was the most frequently used, accounting for approximately 60% of the total use (Table 2). Great Britain was – together with Ireland – the dominant user of urea, but reported that DOT will gradually replace urea in the near future. DOT, accounting for 2% of the total use in terms of area treated, was also used in France and Sweden, although not to the same extent as *P. gigantea* (Sweden) or urea (France).

Table 2. The use (treated area, ha yr^{-1}) of *Phlebiopsis gigantea*, urea and disodium octaborate tetrahydrate (DOT) in the survey. The figures of area treated with each compound are calculated from estimated percentage values, making the figures appear more exact than they probably are.

Country		**Control agent**	
	P. gigantea	**Urea**	**DOT**
Denmark	3 850	1 650	0
Finland	11 400	600	0
France	0	240	60
Great Britain	1 380	67 620	0
Ireland		18 450	
Norway	380	20	0
Poland	70 010	0	0
Sweden	31 545	0	3 505
Sum	*118 565*	*88 580*	*3 565*
Percentage of total treated area	*56%*	*42%*	*2%*

The degree of mechanization in stump treatment was 80% or more, with the exception of Poland where all stump treatment was carried out manually (Table 3). From Denmark, Finland, Ireland, Norway and Sweden, at least 95% mechanized treatment was reported. Overall, and including Poland, approximately 60% of the stump treatment was carried out with mechanized methods. If Poland is excluded from the comparison, at least 88% of all treatments would have been carried out mechanically. No information was available from France on this topic.

Table 3. The degree of mechanization in stump treatment (no information from France on this issue).

Country	**Area treated per year**		**Mechanized treatment**	
	thinning	**Clearfell**	**%**	**hectares**
Denmark	5 000	500	>95%	>5 225
Finland	9 000	3 000	>95%	>11 400
France	300	0		
Great Britain	60 000	9 000	80%	55 200
Ireland	9 950	8 500	100%	18 450
Norway	400	0	>95%	>380
Poland	70 000	10	0%	0
Sweden	35 000	50	>95%	>33 298
Sum	*189 650*	*21 060*		*>123 953*

Two countries, Finland and Poland, have reported that there are subsidies available for stump treatment. In Finland, private forest owners are compensated for the cost of material, while in Poland the State forest districts pay for costs of material, application and monitoring/follow-up.

The costs for treatment varied between 0.2 and 2 euro per m^3 solid under bark (Table 4). Locally, the cost depends on stand conditions, nature of thinning or final felling etc. Some of the countries did not separate costs for thinning and final felling. In Ireland, the harvesting contractor is not paid extra for stump treatment, but the costs which have been reported cover the supply of pre-dissolved urea solutions to the contractors. In terms of a weighted mean value, stump treatment costs – as expressed by the country representatives – are about 1.2 euro per m^3sub in thinning and 0.4 euro in final felling.

Table 4. The cost for stump treatment, including the cost for material and application, euro per m^3 solid under bark.

Country	**Cost, euro/m^3 sub**	
	thinning	**clearfell**
Denmark	1.35	1.35
Finland	0.75	0.4
France		
Great Britain	0.5	0.5
Ireland	0,2	0,2
Norway	1.35	
Poland	2	
Sweden	1.3	0.33
Weighted Mean	*1.22*	*0.38*

DISCUSSION

The country-wise estimates on which this survey relies inevitably include some uncertainties. The quantities of materials used and the number of hectares treated are seldom accurately reported, but must be estimated from related facts and knowledge such as the volumes of control agents deployed. Nevertheless, the estimates are sufficiently realistic to allow observations to be made on the approximate levels and trends nationally and on a European level.

Calculating the area to be treated annually has some practical difficulties, and may not necessarily reflect the whole picture. In terms of area treated, thinning accounted for 93% while the thinning proportion in terms of harvested volume should be about 65% (assuming a yield of 50 m^3sub per hectare in thinning and 250 m^3sub per hectare in final felling). Differences between countries in the materials used depend on several factors, e.g. historical background, efficacy on specific tree species and legislation. Urea has a long history in stump treatment, but has lost ground in many countries. In Great Britain, which is one of the two remaining significant users of urea, the intention is to abandon urea because of its poor efficacy on Sitka spruce. In addition, urea is transported in bulk solution, which makes the logistics more difficult and expensive in comparison with *P. gigantea* and DOT. *P. gigantea* is a natural coloniser of pine stumps, and it is most frequently used on pines in Great Britain and Poland. In Finland, however, some strains of *P. gigantea* were discovered that are successful colonizers also on spruce stumps (Korhonen et al. 1994). This strain was formulated into a product, "Rotstop", which is used in Denmark, Finland, Norway and Sweden. In Poland, a biological product is used with sawdust as a substrate ("PG-IGL"), whereas in Great Britain, the "PG Suspension" is formulated with sucrose suspension packed in small sachets. Details about these products are given in Pratt et al. (2000).

DOT has been subject to decreased use in Sweden due to its classification as a pesticide, which disqualifies it for use in woodland certified according to Swedish FSC criteria. In France, where DOT is also used, albeit in small quantities, the compound is classified as a fertiliser, and is unregulated for forestry purposes. Until the European Union has harmonized the legislation on pesticides over all countries, the member states' national law will state what is legal or not. Since there are few approved and suitable control agents available, and a low probability for further products to be developed, the forest sector must ensure that those available are used carefully, effectively and responsibly.

Stump treatment represents an investment for the future, and must render the expected payback on the investment (be cost-effective). Assumed the extraction volumes given above, the countries in the survey invest at least 13 million euro annually in stump treatment. In Great Britain, it is no longer recommended to carry out stump treatment on sites where the risk from the disease is low. In Finland, Great Britain and Denmark treatment is recommended and carried out in final felling as well as in thinning. In contrast, Sweden enjoys an ongoing discussion about whether to begin stump treatment in final felling or not. The problem is that the longer the rotation period, the harder it is to get a proper payback on the investment from treating stumps in the final felling, given the net present value of felling assuming a specific interest rate. Preliminary calculations indicate stump treatment in final felling to be profitable in stands with the relatively short rotation periods common in southern Sweden, but not on the Norway-spruce sites in the north where the rotation periods are too long. Regardless of these disagreements, there are sufficient areas that are currently not treated, as final-felling stands in southern Sweden and thinning stands in the north, to indicate that an overall increase in area treated can be expected.

There are examples of incentives for stump treatment. In Finland, the private forest owners are compensated for the cost of protectant, in thinning as well as in final felling. In Poland, the subsidies cover the cost for protectant, application and monitoring.

In addition to strict economics, there are also some aspects concerning the amount of inoculum that can develop in the forests in the long term. To reduce the increase of inoculum, treatment of stumps is necessary. Here, the state forests could play an important part to ensure healthy stands for the future. This has been the case in Denmark, Great Britain and Poland, where stump treatment has been compulsory on state-owned forest land.

In spite of the relatively large extent of stump treatment in Europe there are several issues to be addressed further:

- The technology and methods for applying stump treatment in a wide range of tree and stand conditions exists, but need to be further improved. There is considerable scope to reduce the consumption of protectant in order to reduce costs and to facilitate the logistics of supply. Using properly designed equipment can help here, since retro-fitted systems can be very inefficient.
- Motivation and training is essential in order to accomplish high standards of stump treatment. Without understanding the motives, contractors and forest workers object to apply a treatment that increases their costs and causes them extra work.
- Assuring a good quality of work includes follow-up routines. Stump treatment should be an integral part of each forest company's follow-up and monitoring programme.
- Harmonizing EU directives into national legislation would facilitate the use of the three different control agents that are available. A natural competition with fair conditions for all manufacturers and suppliers of stump treatment material is essential to provide effective products to reasonable costs.

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STUMP TREATMENT EXPERIMENTS AGAINST *HETEROBASIDION* IN THE ITALIAN ALPS

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SUMMARY

The efficacy of urea, *Trichoderma* sp. and different strains of *Phlebiopsis gigantea* in controlling spore infection of *Heterobasidion* in Norway spruce stumps was investigated in two experiments carried out in a spruce stand heavily infected by *Heterobasidion annosum s. str.* and *H. parviporum*. In the first experiment, made in late spring during rainy weather, the protecting agents were applied to freshly-cut stumps. The Rotstop preparation proved most effective (efficacy 70–90%) and an Italian strain of *P. gigantea* showed some efficacy, while urea, *Trichoderma* and a *P. gigantea* strain from Germany failed totally. The second experiment was carried out in the autumn in stem pieces of Norway spruce. The pieces were first incubated in the forest, later in the greenhouse. In this experiment, urea was very effective (90–100%), while *Trichoderma* and Rotstop were less effective (70–80%), and six *P. gigantea* strains from Italy were unsatisfactory (0-60%). *P. gigantea* strains grew very weakly in the stem pieces, probably as a result of low initial temperature and later high water content in the wood.

Keywords: biological control, stump treatment, *Heterobasidion annosum*, *H. parviporum*, *Phlebiopsis gigantea*, *Trichoderma* sp., Rotstop, Norway spruce

INTRODUCTION

Chemical and biological control of *Heterobasidion* in coniferous forests has been focused mainly to preventing the spore infection on stump surfaces since the early establishment of the mycelium is the most susceptible stage to the action of antagonists (Holdenrieder and Greig 1998). Among low-toxicity chemicals, urea and borates have been found to be effective (Pratt 1994, 1996). The efficacy of *Phlebiopsis gigantea* (Fr.) Jül. on pine stumps was reported by Rishbeth (1963), and later experiments carried out in Finland and other countries have indicated that this fungus can be effective also in Norway spruce stumps (Korhonen et al. 1994, Thor and Stenlid 1998, Pratt et al. 2000, Soutrenon et al. 2000). In Trentino, as in most temperate coniferous forests, *P. gigantea* is a common inhabitant of pine and spruce stumps, and during warm rainy autumns it fruits quite abundantly on them. A rapid increase in the number of sites infected by *Heterobasidion* root rot in Italy together with new requirements for "environmentally-safe" pesticides has stimulated research into potential biological control agents to prevent the spread of this pathogen.

The aim of this study was to compare the efficiency of the following treatments against a heavy natural infection of Norway spruce stumps by *Heterobasidion* in the Italian Alps: 1) isolates of *P. gigantea* originating from the Alps, 2) *P. gigantea* preparation Rotstop® (Kemira Agro OY), which includes a *P. gigantea* strain isolated from Finland, 3) strains of *Trichoderma*, and 4) urea.

MATERIAL AND METHODS

Stump treatment experiment. A stump treatment experiment was performed on May 27, 1998, in a pure even-age plantation of Norway spruce about 35 years old, situated at 900 m a.s.l. in Trentino (Eastern Italian Alps), established on a former pasture land. The stand was heavily infected by *Heterobasidion*. Basidiocarps of the fungus were numerous, and the place looked suitable for a stump treatment experiment, providing a heavy natural basidiospore infection. The following treatments were applied to the stumps: 1) Rotstop, 2) *P. gigantea* 94135 (Germany, Munich), 3) *P. gigantea* 97099 (Italy, Trentino), 4) *Trichoderma* sp. 94268 (isolated from pine wood in Finland), 5) urea solution (30%), 6) control (sterile water). The treatment was applied immediately after cutting a tree. Spore concentration in *P. gigantea* suspensions was ca. 10 000 spores/ml and in *Trichoderma* suspension ca. 40 000 spores/ml. The amount of the solution applied to each stump was ca. 10 ml/dm^2, corresponding to a fluid layer of 1 mm on the stump surface. In the case of urea, 20 ml/dm^2 was applied. It was raining when the treatments were carried out, and also two following days were rainy. The precipitation during these three days was 15.4, 7.0 and 15.8 mm, respectively. The temperature during the following week varied between +4 and +21°C.

The stumps were sampled after two vegetation periods, at the end of September 1999. Two ca. 3 cm thick discs were cut from the stumps. The upper disc was discarded, the lover one was washed in running water, incubated for 7 days in plastic bags, and investigated on both sides for the presence of *Heterobasidion* conidiophores. Also the area occupied by *P. gigantea* was approximately determined on the basis of characteristic orange-brown colour.

Experiment in stem pieces. This experiment was made relatively late in the autumn, in October 6, 1999, using pieces cut from fresh Norway spruce stems. Four healthy spruce stems with stump diameter ca. 20 cm were selected from a site free from *Heterobasidion* root rot and carried to the experimental forest. The basal part of each stem was cut to pieces, 20 cm long, 27 pieces from each stem. After cutting, the upper surface was divided in two symmetrical halves. One half was sprayed with the protecting agent while the other half was covered. One to two hours later the whole surface was sprayed with sterile water. The protecting agents were: 1) urea 30%, 2) *Trichoderma* 94266 (isolated from pine wood in Finland), 3) Rotstop, 4) *P. gigantea* 97092 (Italy, Trentino), 5) *P. gigantea* 97097 (Trentino), 6) *P. gigantea* 97099 (Trentino), 7) *P. gigantea* 97104 (Trentino), 8) *P. gigantea* 95051 (Italy, Tuscany), 9) *P. gigantea* 95042 (Italy, Abruzzo). The spore concentrations were adjusted to ca. 5 000 spores/ml for each *P. gigantea* strain (including Rotstop) and to ca. 20 000 spores/ml for *Trichoderma*. The number of repetitions was 12 (three repetitions in each spruce stem).

After treatment, the stem pieces (spatially randomised) were left standing in a humid place in the forest. The weather during the treatment was sunny and dry, and six following days also were rainless. The stem pieces and the soil around them were sprayed with clean water from time to time. The temperature during this period varied between +2 and +13°C. After three weeks, at the end of October, the stem pieces were removed from forest and placed for five weeks in a controlled greenhouse with humidity of 80% and temperature of 25°C. The stem pieces were sampled in December 1999. Two discs were cut; the first disc (3 cm thick) was discarded, the second disc (5 cm thick) was investigated on both sides as described above. The efficacy of the treatment was calculated by comparing the area occupied by *Heterobasidion* on treated and untreated halves of each disc surface.

RESULTS

Stump treatment experiment. The experimental spruce stand proved to be more infected by *Heterobasidion* than expected: the frequency of trees with butt rot was ca. 80%. Two species were present in about equal frequencies: *H. annosum* (Fr.) Bref) *s.str.* and *H. parviporum* Niemelä & Korhonen. Altogether, 14 *Heterobasidion* isolates from diseased trees were identified, seven of them belonged to the former and seven to the latter species. Because only sound stumps could be used for the stump treatment experiment, the number of stumps within each treatment remained low. Moreover, a part of the discs had to be discarded because of apparent

Heterobasidion infection coming from the roots. The final number of accepted stumps in different treatments was: control 19, Rotstop 21, urea 14, *P. gigantea* (94135) 13, *P. gigantea* (97099) 12, and *Trichoderma* (94268) 11.

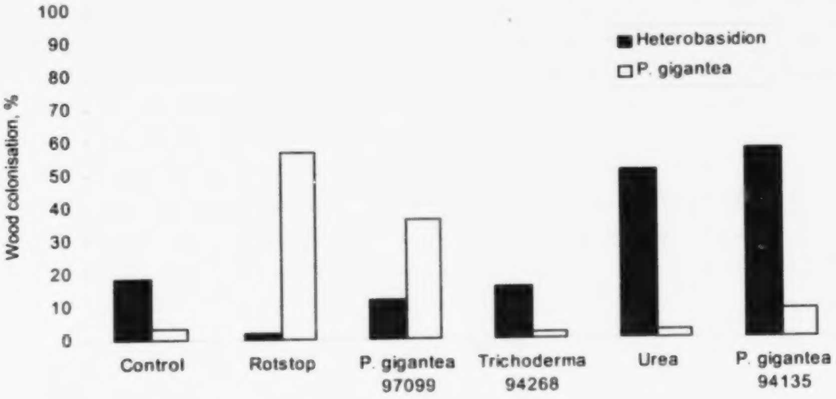

Figure 1. Wood colonised by *Heterobasidion* spp. and *P. gigantea* in Norway spruce stumps at the depth of ca. 6 cm from stump surface. The results were calculated as percentage of infected wood of total wood on disc surfaces (all discs counted together).

At the depth of 6 cm from the stump surface, *Heterobasidion* had infected 18.6% of total wood in untreated control stumps (Fig. 1); mean amount of infected wood per stump was 20.6%, and the number of infected stumps 52.6%. Rotstop treatment proved relatively effective against *Heterobasidion* infection but the treatments with urea, *P. gigantea* 94135 and *Trichoderma* 94268 rather favoured than reduced the infection, although the result may not be statistically significant. The efficacy of different treatments against *Heterobasidion* infection was as follows (calculated in three different ways):

Table 1. Efficacy of different treatments against *Heterobasidion* measured in percentage of total wood, average of infected wood and number of infected stumps.

	Infected wood of total wood	**Mean infected wood per stump**	**Number of infected stumps**
Treatment	**Efficacy**	**Efficacy**	**Efficacy**
Rotstop	90.6%	85.4%	70.0%
P. gigantea 97099	35.5%	38,9%	-10.8%
Trichoderma 94268	16.3%	-22.5%	-3.6%
Urea, 30%	-173.2%	-130.6%	-35.7%
P. gigantea 94135	-208.2%	-162.7%	-46.2%

Experiment in stem pieces. In this experiment, the spore infection by *Heterobasidion* was relatively low; only 4–5% of the wood in untreated control halves of the stem pieces was colonised by *Heterobasidion* (at the depths of 3 and 8 cm from the surface). The colonies were generally small in size. The growth of *P. gigantea* was even weaker; even in treated halves of the stem pieces the mean colonisation was only 3.5%. The efficacy of different treatments against *Heterobasidion* infection is shown in Fig. 2. In striking contrast to the stump treatment experiment, the treatment with urea proved to be most effective (efficacy 90–100%). *Trichoderma* and Rotstop were distinctly weaker (70–80%). *P. gigantea* isolates from Italy were even weaker (0–65%).

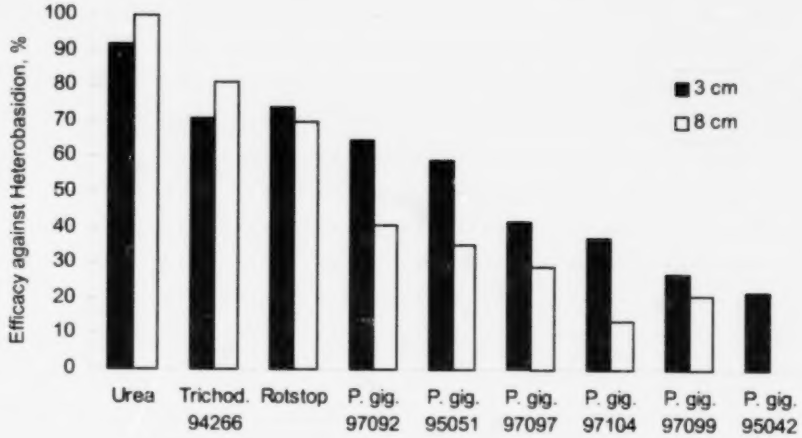

Figure 2. Efficacy of different treatments against *Heterobasidion* infection in stem pieces of Norway spruce. Two depths, 3 and 8 cm, from the treated surface were investigated. The efficacy of *P. gigantea* strain 95042 at the depth of 8 cm was slightly negative.

DISCUSSION

The amount of precipitation and the water content of wood apparently had a major impact on the results obtained in these two experiments. It was raining when the stump treatment experiment was made in spring 1998, and the rain continued during the following days. This is probably the main reason why the urea treatment failed totally in this experiment. Although urea generally works well in stump treatments, occasional failing of urea has been reported also earlier (e.g. Schönhar 1977, Pagony 1980). Rotstop was quite effective in this experiment, and in stumps treated with Rotstop *P. gigantea* colonised more than 50% of the wood. The two *P. gigantea* isolates originating from the Alps as well as *Trichoderma* sp. did not work satisfactorily.

In the second experiment, the treatments were made to stem pieces of spruce. To obtain a natural spore infection by *Heterobasidion*, the pieces were first kept in the forest for three weeks, but later the incubation was carried out in the greenhouse with high relative humidity. At the end of the experiment the wood had a higher water content than is usual. In these wet pieces of spruce wood, the efficacy of urea against *Heterobasidion* was very good. The *Trichoderma* strain also proved to be relatively effective; it was as effective as Rotstop. The Italian isolates of *P. gigantea* were weaker. The high moisture content of wood apparently inhibited the growth of all the *P. gigantea* strains used in the experiment. Unexpectedly, the growth of *Heterobasidion* was not inhibited in the same degree, although in general *P. gigantea* and the *Heterobasidion* species seem to have very similar requirements as regards moisture content of spruce wood (K. Korhonen, unpublished results). It is also possible that the relatively low temperature at the beginning of the experiment slowed down the colonisation by *P. gigantea*.

In conclusion, continuous rain during and after the stump treatment may have a great impact on the efficacy in case urea is used as a protecting agent. The rain at the time of application does not seem to affect so much on the efficacy of treatment with *P. gigantea*, but a long-lasting high water content of wood inhibits the growth of this fungus, and may decrease its efficacy against *Heterobasidion*. The results also indicate big differences in the capacity of different *P. gigantea* strains to control spore infection of *Heterobasidion* in Norway spruce stumps.

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MICROBES INHABITING *PICEA* WOUNDS AND THEIR ANTAGONISM TO *HAEMATOSTEREUM SANGUINOLENTUM*

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SUMMARY

The Boreal Mixedwood Forest is the most productive forest region in Ontario. Prescriptions to maintain advanced growth in these stands call for partial cutting, which involves the use of machinery in confined spaces. Wounding of residual trees is unavoidable and often results in infection by decay fungi. Wounds of black and white spruce inflicted when the average air temperature was greater than 0°C (pre-freeze up) or less than 0°C (post-freeze up) were evaluated periodically over 8 months to determine if the ambient temperature at the time of wounding influenced the diversity and establishment of pioneering mycoflora on the wound surface. The size of wounds varied from 0.6 cm^2 to 850.8 cm^2 with the highest proportions of wounds being on the root, stem and butt respectively. Pre-freeze up wounds had the highest level of biodiversity of microorganisms and *Pseudomonas* species comprised the greatest proportion of the populations on these wounds. Yeast species (most commonly *Crytococcus albius* var *albius*) dominated the surface of wounds inflicted during the post-freeze up period. Isolates of *Pseudomonas* and *Trichoderma* species showed the greatest inhibition against the linear growth of *H. sanguinolentum* in *in vitro* studies whereas none of the yeasts demonstrated antagonistic properties. Over the duration of the study decay fungi were isolated more often from post-freeze up wounds than from pre-freeze up wounds. *Poria rivulosa* was the most commonly isolated decay organism.

INTRODUCTION

Partial cutting operations involves the maneuvering of large pieces of equipment in somewhat confined spaces. These operations ultimately result in unavoidable wounds to the residual trees, initiating the complex mechanism often resulting in decay. Wounding of coniferous wood enhances the invasion of bacteria (Kallio 1973, 1974; Roll-Hansen and Roll-Hansen 1980; Hallaksela 1984) and fungi (Isomaki and Kallio 1974; Roll-Hansen and Roll-Hansen 1980; Vasiliauskas et al. 1996). Affected trees are prone to blowdown and breakage at the wound site and if they survive to rotation age their end values are diminished due to the staining and decay in the wood.

The ability of a microbe to function and sustain itself depends on several factors such as suitable substrates, water, oxygen, other gases and temperature (Rayner and Boddy 1988). When trees are wounded a series of events occur that will determine the mycofloral component of the wound face and the succession of conditions that ultimately result in stain and decay. Shigo (1979, 1984) recognized three succession stages. The first includes the physiological processes of the host response to wounding. The second stage of succession occurs when pioneer invaders, primarily bacteria, yeasts and deuteromycetes invade the area and overcome the physiological and chemical barriers of the host. These pioneering microbes are capable of modifying phenolic substances (Shigo and Sharon 1968, 1970; Shortle and Cowling 1978). The final step in the microbial succession is invasion by decay fungi. This is the mode of infection for most decay fungi but some, such as *Haematostereum sanguinolentum*, do not need the wound substrate to be preconditioned by other microbes (Davidson and Etheridge 1963). Also, the season of wounding appears to influence the likelihood of infection by some decay fungi. In earlier studies (Kallio and Hallaksela 1979; Beitzen-Heineke and Dimitri 1981; Hallaksela 1984), *H. sanguinolentum* was observed to infect more frequently in the cooler months of the year.

The purposes of this study were to investigate the diversity of pioneering microbes colonizing wounds of spruce, the influence of temperature on microbial diversity, and the abilities of the various microbes to inhibit *H. sanguinolentum*.

MATERIAL AND METHODS

The study areas were located approximately 120 km northeast of Thunder Bay, Ontario, on the management limits of Bowater Inc. within the Black Sturgeon Forest (49°11.4' N, 88°42.5' W). Root, butt (i.e., wounds in the area of 0 to 20 cm above the soil line) and stem wounds (i.e., wounds higher than 20 cm on the stem) on residual black spruce (*Picea mariana*) and white spruce (*Picea glauca*) trees were selected for sampling. Manual or feller-buncher cutting and skidding during partial cutting operations caused the wounds. Seventy-six wounded trees were sampled in early October when the mean aerial temperature was higher than 0°C and are referred to as pre freeze-up wounds. Twenty-three wounded trees were sampled in late October/early November when the mean aerial temperature was below 0°C and are referred to as post freeze-up wounds. In total, 143 pre freeze-up wounds and 48 post freeze-up wounds on 76 and 23 trees respectively were sampled.

The size of each wound was measured by photographing it in relation to a 76-mm x 127-mm index card. The picture was projected onto a screen and traced with a planimetre. The area of the wound was determined through a magnification factor based on the area of the index card.

The first samples were taken 1 week after wounding. The wound surfaces were wiped with sterile cotton swabs moistened with a sterile 20% glycerol solution. The swabs were then placed in sterile 4-mL plastic vials containing 0.5 mL of sterile 20% glycerol and transported to the laboratory in coolers. They were stored at 4°C and processed within 2 days. The two week, 6-7 week, 5 month and 7-8 month samples were thin wood chips approximately 1.5 cm x 1 cm excised from the wound face with a scalpel sterilized with 70% ETOH. The wood chips were placed in plastic vials containing 0.5 mL of sterile 20% glycerol at 4°C. The 18-19 month samples were wood chips approximately 2 cm x 2 cm x 0.5 cm thick cut from the wound face with a chisel cleaned with 70% ETOH. Each sample was placed in a plastic bag for transporting to the laboratory.

A selective medium was prepared from both black spruce and white spruce wood chips. Black and white spruce stem sections, free of defects and approximately 1.5 m long and 12 cm in diameter, were debarked and chipped on a woodworking jointer. The chips were collected and kept frozen until needed. The moisture content of the chips was determined by drying 100 g fresh weight of wood chips in an oven at 80°C for 18 hr and subsequently placed over CaSO4 in a dessicator until a constant weight was achieved. Wood chips, equivalent to a dry weight of 2000 g, were put into a 6-L flask. Four litres of sterile distilled water, brought directly from the autoclave, were poured onto the wood chips and the infusion was left to steep for 16 hours. The infusion was filtered through Whatman1 filter paper under vacuum and then through 0.45-µm Gelman filter. Eighty grams of sucrose were added to the extract and the volume brought up to 4 L using a volumetric flask and stored at 4°C until needed. Fifteen grams of agar were added to each litre of wood extract-sucrose solution and autoclaved for 15 minutes at 110°C. Twenty mL were dispensed into 100-mm x 15-mm sterile plastic petri dishes. The other isolation media were 1/10 strength TSA (4 g CASO + 15 g agar/L) and PDA (Potato Dextrose Agar). To aid in the isolation of decay fungi, MEA (3% malt extract + 1.5% agar) was supplemented with 5 ppm MBCP (methylbenzamidazole carbamate phosphate).

Isolations were made by first adding 1 mL of sterile 0.1M $MgSO_4$ to the contents of each swab sample vial. The vials were then agitated and the suspension streaked onto three defined media and the spruce extract medium, three replicates per medium. The undiluted liquid contents of the vials containing wood chips were also streaked onto 1/10 TSA to determine if microbes were leached from the wood sample. Colony forming units were assessed only on the swabbed samples. Bacteria and actinomycete isolates were transferred to nutrient agar (NA) and the fungal cultures to PDA. The populations isolated on these media were not tallied in subsequent samples because of their low numbers. Isolation attempts for a broad range of fungi were made after 1 and 2 weeks, 6-7 weeks, 5 months, and 7-8 months. The wood chip samples were cut into two pieces. One half was placed on MEA

+ 10 ppm MBCP to favour isolation of basidiomycetes and the other half on PDA for moulds. Wood chip samples collected 18-19 months after the wounds occurred were plated only on MEA + 10 ppm MBCP. The plates were wrapped with Parafilm7 and incubated in the dark at 25°C.

Microbes isolated on synthetic media were then cultured on the spruce extract medium. Only those that were capable of growing on the spruce extract were selected for identification. Bacteria were first identified as either gram-positive or gram-negative according to the method described by Suslow et al. (1982) and their morphological characteristics on 1/10 TSA and nutrient agar. Further identification was accomplished using the MIDI® software system. Fungi capable of growing on the spruce extract medium were transferred to PDA and incubated at 25°C on a 16-hr light/8 hr dark cycle to encourage conidiogenesis. Fungal colonies that were morphologically and microscopically similar were pooled for identification by reference to standard identification keys. Yeast isolates were identified by the MIDI® software system. Basidiomycetes were identified according to Stalpers (1978).

The *Haematostereum sanguinolentum* isolate used in the study was obtained from a spore cast from fruiting bodies on black spruce originating from the study site. Fungi, bacteria and yeasts were paired with *H. sanguinolentum* to detect antagonism. The fungi were tested on 3% malt agar while the bacteria and yeast were tested on the malt extract-yeast extract-peptone medium of Rose et al. (1980). Five-mm-diameter mycelial plugs were cut from the margin of actively growing cultures and plated with *H. sanguinolentum* 55 mm apart on MEA (3%) in 90-mm petri plates. The plates were incubated in the dark at 25°C for 2 weeks. The interactions between the potential antagonists and the *H. sanguinolentum* were observed and recorded after 2 and 4 weeks. Interactions were classified as formation of an unoccupied aversion zone between the pairs (inhibition), meeting at the confluence zone with or without hyphal massing and/or minimal intermingling (collision), and an interaction where either the *H. sanguinolentum* or the other isolate overgrew the other (overgrown).

Isolates that inhibited the growth of *H. sanguinolentum* and those that overgrew it were selected for further tests. In this second stage *H. sanguinolentum* was grown for 5 days under conditions described above before the antagonists were introduced to the plates. The pairs were incubated as described above and the ability to overgrow or inhibit the growth of *H. sanguinolentum* was measured after the control colonies reached the edge of the plate. All bioassays had three replicates and were repeated three times.

RESULTS

After one week most of the soil and organic material that had been deposited on the wounds from the skidder tires or logs had been washed by rain from the wound surface. Therefore, the microbes living on the wound surface were presumably obtaining sustenance from the tree and not the soil.

The number and ratio of bacteria, actinomycetes, and fungi on one-week-old wounds varied between pre and post freeze-up wounds, wound locations, and media. Random samples of the different classes of microbes isolated from the two spruce species were not significantly different, indicating no relationship between tree species and microbe. Therefore, data sets were combined and are reported as a total population study.

The highest numbers of wounds occurred on the stem (94), roots (64) and butt (33), respectively. The largest wounds were found on roots (mean size 137 cm^2, ranging from 7.5 cm^2 - 851 cm^2), followed by stem wounds (mean size 69 cm^2, ranging from 1 cm^2 - 785 cm^2) and butt wounds (mean size 44 cm^2, ranging from 5.3 cm^2 - 162 cm^2). There was no relationship between wound size and the time they were inflicted.

More bacteria than fungi were isolated from the pre freeze-up wounds than from the post- freeze up wounds, while fungi were more plentiful than bacteria on the post freeze-up wounds. Bacteria populations were greater on root wounds than butt wounds, which in turn had higher populations than stem wounds (Table 1). Yeasts comprised a large proportion of the fungi from the post freeze-up wounds.

Identifications using the plasmalemma fatty acid analysis indicated that there was a complex of bacteria capable of living on the surface of one-week-old wounds. Species of *Bacillus* and *Pseudomonas* comprised the highest proportion of the microflora. A much greater diversity of bacterial species was isolated from the pre freeze-up wounds than from the post freeze-up wounds. *Bacillus sphaeficus*, isolated from the pre freeze-up wounds, demonstrated the highest antagonism to *H. sanguinolentum* (Table 2).

Yeasts numbers and diversity were highest on wounds incurred during the post freeze-up period. *Cryptococcus albidus* var. *albidus* was the most frequently isolated yeast (Table 3). The inhibition test demonstrated that all yeasts were ineffective at inhibiting the growth of *H. sanguinolentum* (data not shown).

Filamentous fungi were more prevalent than yeasts on the pre freeze-up wounds. Sampling period (i.e., pre or post freeze-up) had no significant effect on the diversity of identified species. However, there were also many fungi that failed to form fruiting structures and as such could not be identified. There were more than twice as many fungal isolations from the pre freeze-up wounds than from the post freeze-up wounds. *Trichoderma polysporum* was frequently isolated from pre freeze-up wounds (Table 4).

Inhibition tests demonstrated that only a few of the fungi isolated were capable of inhibiting *H. sanguinolentum*. The *Penicillium* species were the most inhibitory, producing distinct inhibition zones between the cultures whereas the *Trichoderma* species overgrew the *H. sanguinolentum* colony and eventually digested the mycelia (Table 5).

The highest incidence of isolation of decay fungi was from the samples taken 7 to 8 months following wounding. *Poria rivulosa* (Berk. & Curt.) Cooke was the most commonly isolated decay fungus. A greater diversity of decay fungi were isolated from wounds incurred during post freeze-up (11 spp.) than during pre freeze-up (8 spp.) (Table 6).

DISCUSSION

Levy (1975) observed that wood contained appropriate nutrients for primary wood invasion by bacteria. The present study demonstrated that the micro environment at the surface of a wound would determine the initial microflora. Also, by waiting 1 week after wounding before attempting to isolate microbes we believe that most of the isolated microbes were surviving on nutrients provided solely by the tree. Selection of microbes capable of surviving on the wound surface was further refined through the use of the spruce extract medium. He number of bacteria colonies isolated on synthetic medium were very high but were substantially reduced when grown on the wood extract medium. Kallio's (1973) medium was not used because it was thought that the yeast extract and trypton in that medium might select for bacteria incapable of living only on the nutrients available on the wound surface.

Etheridge (1969) found a prevalence of *H. sanguinolentum* in wounds inflicted at lower temperatures. He suggested that the dominance of *H. sanguinolentum* of fresh wound surfaces was due to the properties of the substrate, especially at low temperatures, but the reasons why were not thoroughly studied. However, the reason could be that different microbial populations become established on wounds inflicted at different times of year, under different growing conditions, especially temperature. In the pre freeze-up period, bacteria and fungi, which were more active in inhibiting the growth of H. sanguinolentum, dominated the wounds whereas yeasts primarily colonized the post freeze-up wounds. Although yeasts have been shown to possess anti-fungal properties, (Payne et al. 2000; Janisiewicz and Bors 1995; Burr et al. 1995; Walker et al. 1995) the isolates obtained in this study were not antagonistic towards *H. sanguinolentum*.

The diversity of bacteria isolated in this study was much greater than previously reported, probably because previous research concentrated on the bacteria that are capable of inhabiting the inner wood. Nevertheless, in this study *Pseudomonas* and *Bacillus* spp. were the bacteria species most commonly isolated from the wound surface. This is similar to the findings of Hallaksela et al. (1991) and Hallaksela and Salkinoja-Salonen (1992) who found that these genera were the major types which inhabited the inner portions of the wood of Norway spruce. Przybyl and Żłobińska-Podejma (2000) isolated *Pseudomonas* species most commonly from discoloured wood of *Betula pendula* Roth. These species are present in the soil (Holt et al. 1994) and could enter the inner wood through minute wounds in the roots or be deposited on the wound via splashing from the machinery tires. Some of the isolated species may be endophytes that became exposed when the bark was removed.

Kallio (1974) isolated bacteria that were antagonistic to *H. sanguinolentum* and other decay fungi but did not identify them. The antagonism of these bacteria to *H. sanguinolentum* is similar to results of tests against *Armillaria ostoyae* in an earlier study (Dumas 1992). The mode of the antagonistic action is through the production of antifungal compounds and siderophores (Dumas and Strunz 1997), compounds that could possibly be produced on these sites.

The diversity of fungal species was high in the initial isolations but as the wounds aged it decreased. The initial large populations and diversity could be similar to the situation with the prokaryotes. The initial abundance and diversity of microbes would include many that can metabolize only simple sugars. As the wound ages there would be a gradual succession to organisms that can utilize more complex organic compounds for nutrition, similar to soil microbial adaptation as described by Panikov (1999). In the present study deuteromycetes comprised the bulk of the populations in the wounds of black and white spruce at all sampling periods. Roll-Hansen and Roll-Hansen (1980b) found a higher proportion of ascomycetes than deuteromycetes in wounds on *P. abies* in Norway whereas Michalopoulos-Skarmoutous (1987) observed a greater number of deuteromycetes in the same tree species in Greece. The variations could be due to differences in geographical locations and wound microsites. A large proportion of the fungi isolated in the current study did not inhibit the growth of *H. sanguinolentum*. The *Trichoderma* species inhibit by acting as a mycoparasite and through the release of anti-fungal metabolites (Dumas and Strunz 1997). Species of *Penicillium* were very antagonistic to *H. sanguinolentum* and the production of a clear inhibition zone indicated the production of antibiotic-like compounds. This is a very common method of antagonism utilized by different species of *Penicillium* (Land and Hult 1987). It is interesting to note that *Beauveria bassiana*, an entomopathogen, (Samson et al. 1988) suppressed the growth of *H. sanguinolentum*.

Stem decay fungi were isolated much later after wounding occurred and were found more commonly on post freeze-up wounds. Roll-Hansen and Roll-Hansen (1980a) observed a higher recovery of *H. sanguinolentum* from wounds inflicted in May and September than those caused during July and December, but overall the occurrences of hymenomycetes was not influenced by the wounding period. The discrepancy in results is most likely influenced by the differences between inoculum potential of the decay fungi and tree species.

We were unable to continue to monitor the changing diversity of the microbial communities within the wood, first because many of the sampled trees died (primarily due to *Armillaria* and windthrow) within 2-5 years after the partial cut, and finally because the experimental site was destroyed by wildfire in the spring of 1999.

Table 1. Mean number of colony forming units of bacteria, actinomycetes, and fungi isolated on 1/10 TSA, spruce extract agar and PDA from 1-week-old wounds on roots, butts and stem of spruce inflicted pre freeze-up and post freeze-up.

	1/10 TSA			Spruce extract agar			PDA		
Wound	**Bacteria**	**Actinomycete**	**Fungi**	**Bacteria**	**Actinomycete**	**Fungi**	**Bacteria**	**Actinomycete**	**Fungi**
pre freeze-up root (54)[a]									
avg	68.6	13.5	3.3	46.7	4.4	8.6	22.6	10.7	5.4
b:f ratio[b]	24.8:1			5.9:1			6.2:1		
butt (23)									
avg	27.3	4.6	5.8	16.5	0.1	6.4	14.5	2.7	6.5
b:f ratio	5.5:1			2.6:1			26.5:1		
stem (66)									
avg	12.4	0.2	3.9	5.1	0.1	4.8	8.5	1.0	4.7
b:f ratio	3.2:1			1.1:1			20.1:1		
post freeze-up root (10)									
avg	20.4	25.8	22.4	4.5	0.0	94.2	30.5	8.0	74.7
b:f ratio	2.1:1			0.04:1			0.5:1		
butt (10)									
avg	7.8	1.0	26.8	1.3	0.0	54.2	5.5	1.2	45.3
b:f ratio	0.33:1			0.02:1			0.15:1		
stem (28)									
avg	6.7	0.5	10.1	7.2	0.0	49.8	5.2	0.2	30.8
b:f ratio	0.7:1			0.14:1			0.17:1		

[a] Number of wounds.

[b] Ratio of bacterial to fungal colony forming units.

Table 2. Diversity of bacteria isolated from wound surfaces of spruce at pre freeze-up and post freeze-up and their abilities to inhibit the linear growth of *H. sanguinolentum*.

Wound location	Pre freeze-up		Post freeze-up	
	Bacteria	**% inhibition (Range)**	**Bacteria**	**% inhibition (Range)**
Root	*Arthrobacter illicis* (1)[1]	45.8	*Bacillus mycoides* subgroup A (1)	42.2
	Bacillus longisporus (2)	4.1-8.4	*Norcardia restrictata* (1)	64.8
	Bacillus mycoides subgroup A (17)	36.7-68.5	*Pseudomonas chlororaphis* (3)	70.8-71.1
	Bacillus pasteurii (3)	0	*Pseudomonas fluorescens* Biotype C (1)	69.6
	Bacillus sphaeficus (3)	81.0-86.6	*Pseudomonas fluorescens* Biotype F (1)	64.5
	Bacillus thuringiensis (8)	58.3-68.3	*Pseudomonas putida* Biotype A (1)	67.3
	Cedecca davisae (1)	35.9	*Pseudomonas putida* Biotype B (3)	64.5-69.6
	Cellulomonas turbata (1)	2.9	*Stenotrophomonas maltophilia* (1)	36.9
	Curtobacterium flaccurnfaciens (1)	2.5	*Rahnella aquatilis* (1)	0
	Klebsiella pneumonic, ozaenae (1)	0		
	Micrococcus kristinae (4)	5.5-41.7		
	Norcardia globerula (1)	65.9		
	Norcardia asteroides subgroup A (3)	53.3-64.7		
	Paenobacillus polymxa (1)	2.1		
	Pseudomonas chloroaphis (7)	62.2-73.3		
	Pseudomasfluorescens Biotype C (11)	55.8-73.6		
	Pseudomonas fluorescens Biotype G (1)	69.6		
	Pseudomonas putida Biotype A (1)	67.1		
	Pseudomonas putida Biotype B (5)	68.5-79.9		
	Pseudomonas chloroaphis (7)	62.2-73.3		
	Pseudomonas marginalis (1)	63.3		
	Pseudomonas savastanol pv fraxinus (1)	68.3		
	Rathayibacter rathayi (1)	0		
	Rhodococcus fasicans subgroup B (2)	62.2-70.8		
	Rhodococcus equi subgroup B (1)	24.7		
	Serratia phymuthica (1)	0		
	Stenotrophomonas maltophilia (2)	43.1-49.8		
Butt	*Bacillus circulans* (1)	3.3	*Bacillus mycoides* subgroup A (2)	39.4-42.8
	Bacillus pasteurii (1)	69.6	*Pseudomonas putida* Biotype A (1)	24.1
	Bacillus thuringiensis (4)	63.6-74.5		
	Panotea agglomerans (1)	4.6		

	Pseudomonas chlororaphis (1)	49.8		
	Pseudomonas fluorescens Biotype C (8)	55.7-76.1		
	Pseudomonas putida Biotype B (2)	69.6-68.4		
	Rhodococcus fasicans subgroup B (1)	74.1		
	Staphylococcus saprophyticus (1)	0		
Stem	*Bacillus cereus* (3)	43.0-64.5	*Bacillus mycoides* subgroup A (1)	53.2
	Bacillus mycoides subgroup A (3)	36.7-51.9	*Bacillus pasteurii* (1)	37.8
	Bacillus pasteurii (7)	43.1-67.1	*Pseudomonas chlororaphsis* (1)	69.6
	Bacillus thuringiensis (4)	62.0-68.3		
	Paenibacillus macerans subgroup S (1)	0		
	Paenibacillus pabuli (7)	73.9		
	Paenibacillus polymxa (1)	0		
	Pseudomonas chlororaphis (7)	60.7-78.5		
	Pseudomonas fluorescens Biotype C (1)	50.4		
	Pseudomonas putida Biotype B (3)	65.9-79.7		
	Rabnella aquatilis (1)	0		
	Unknown (1)	0		

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[a] Number of strains isolated and tested.

Table 3. Frequency of occurrence and diversity of yeasts isolated from spruce wound surfaces at pre freeze-up and post freeze-up periods.

Pre freeze-up	**Post freeze-up**
Cryptococcus albidus var. albidus (4)[a]	*Candida auringiensis* (1)
Candida cacaoi (1)	*Candida blankii* (1)
Candida zeylanoides (1)	*Candida cacaoi* (3)
Cryptococcus neoformans subgroup B (2)	*Candida deserticola* (1)
Rhodotorula rubra (1)	*Candida guilliermondii* (1)
Sporobolymces salmonicolor (1)	*Candida hydrocarbofumarica* (1)
No Match (3)	*Candida norvegica* (3)
	Candida philyla (1)
	Candida silvicultrix (1)
	Candida sake (1)
	Candida zeylanoides (16)
	Cryptococcus albidus var. *albidus* (24)
	Cryptococcus neoformans subgroup A (2)
	Cryptococcus neoformans subgroup B (3)
	Cryptococcus terreus (6)
	Geotrichum candida (1)
	Geotrichum candidum (8)
	Hansenula anomala (1)
	Rhodotorula minuta subgroup A (2)
	Rhodotorula minuta subgroup B (1)
	Rhodotorula rubra (3)
	Sporobolomyces salmonicolor (4)
	Trichosporon beigelii subgroup A (1)
	No Match (36)

[a] number of occurrences

Table 4. Deuteromycetes isolated from wounds on spruces during pre freeze-up and post freeze-up periods.

Species	Time of wounding					
	Pre freeze-up			Pre freeze-up		
	Root	Butt	Stem	Root	Butt	Stem
Alternaria spp.	6[a]	4	18	1	2	5
Aspergillus versicolor	-[b]	-	-	-	-	1
Aureobasidium pullans	3	4	8	1	2	3
Beauveria bassiana	-	-	1	1	-	-
Capitorostrum asteridiellae	-	-	-	-	-	1
Cephalosporium sp.	-	-	1	-	-	-
Chalara cylindrica	1	-	-	-	-	-
Chlaunopycnis alba	-	1	-	-	-	-
Cladosporium spp.	13	6	20	2	1	14
Cladosporium herbarum	-	-	1	1	-	1
Cylindrocarpon spp.	-	1	4	-	-	1
Cytospora spp.	4	2	8	-	1	6
Epicoccum nigrum	8	7	17	2	3	2
Fusarium spp.	3	-	11	2	-	3
Helicoma dennisii	-	-	-	-	-	1
Hormonema type	13	11	24	4	7	33
Lichenoconium spp.	-	-	3	-	-	-
Mycelia sterilia	17	20	67	6	11	39
Oidiodendron griseum	-	-	3	-	-	1
Paecilomyces farinosus	10	6	9	1	1	3
Paecilomyces spp.	10	6	5	-	-	-
Penicillium spp.	42	19	16	9	6	13
Phaeococcus spp.	3	-	8	-	2	-
Phialophora spp.	2	-	-	1	1	-
Phoma spp.	9	4	14	3	5	17
Phoma chrysanthemicola	1	-	1	-	-	-
Phomopsis spp.	1	-	7	3	1	2
Sporotrichum sp.	6	-	-	-	-	-
Taeniolina centaurii	1	2	7	-	2	6
Trichoderma harzianum	3	1	-	1	-	1
Trichoderma polysporum	17	4	3	2	-	3
Trullula sp.	-	-	-	1	-	-
Umbelopsis versiformis	-	-	-	1	-	-
Valsa friesii	1	-	2	-	-	1
Zythiostroma pinastri	8	5	1	1	-	2
Total	**172**	**103**	**259**	**43**	**45**	**159**

[a] Number of isolations

[b] Not detected

Table 5. Fungi inhibitory to the growth of *Haematostereum sanguinolentum*, isolated from wounds on black and white spruce.

Wound location	Pre freeze-up		Post freeze-up	
	Species	**% inhibition**	**Species**	**% inhibition**
Root	*Penicillium glabrum*	68.5	Unknown 3	54.7
	Penicillium lividum	76.7	*Beauveria bassiana*	61.7
	Trichoderma polysporum	100.0	*Trichoderma polysporum*	100.0
Butt	*Penicillium franisosus*	59.3	*Trichoderma harzianum*	100.0
	Chlaunopncinis alba	72.0		
	Penicillium spinolosum	71.1		
	Penicillium soppii	65.8		
	Penicillium steckii	63.3		
	Trichoderma harzianum	100.0		
Stem	*Beaveria bassiana*	72.1	*Aspergillus versicolor*	66.9
	Unknown 4	45.1	*Penicillium brevicompactum*	66.3
	Unknown 5	71.5	*Zythrostroma pinastrii*	75.8
			Unknown 2	61.9

Table 6. Decay fungi isolated from wounds of spruce caused during pre freeze-up and post freeze-up.

Decay fungus	Pre freeze-up wounds (%)	Post freeze-up wounds (%)
Cryptoporus volvatus	0	2
Gloeophyllum protractum	0	2
Haemostereum sanguinolentum	0	2
Hymenochaetae tabacina	1.9	0
Odontia corrugata	1.9	6
Phelbia subserialis	1.9	6
Poria rivulosa	3.8	20
Poria vaillantii	0.97	4
Sistotrema brinkmannii	2.9	4
Strecchericium ochraceum	0	2
Unknown A	0	2
Unknown B	0	2
Unknown C	0.75	0
Unknown D	0.75	0

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COSTS AND EFFECTS OF BIOLOGICAL CONTROL OF ROOT ROT IN POLAND

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Root rot caused by *Heterobasidion annosum* (Fr.) Bref. has been one of the most significant pathological and economic problems in Polish forests for many years. Serious damage has been specifically observed in Scots pine and Norway spruce stands cultivated on soils formerly under agricultural use. After World War II, former agricultural lands, pastures, grasslands and wastelands were continually afforested mostly with Scots pine (*Pinus sylvestris)*, Norway spruce (*Picea abies)*, birch (*Betula* spp.), larch (*Larix decidua*) and alder (*Alnus* spp.) Approximately 1 156 000 ha of former agricultural land and farm abandoned sites were afforested during the years 1947-1997 and 704 000 ha of such forests were state owned and managed by the government (State Forests). Afforestation increased forest cover in Poland from 20.8% in 1946 to 28.0% in 1996.

The first significant damage (13 700 ha) was recorded in cultures and plantations in the 1950's and 1960's. Within 30 years (by 1976) the progress of the disease was recorded in 122 000 ha of stands older than 20 years. Within 50 years (by 2000), damage due to *H. annosum* in tree crop was found in stands of all age classes covering an area of 201 900 ha of state forests (Fig. 1). In 1997, some forest divisions reported the high incidence of the disease in affected stands (5-6% of the forested area). In some forest districts, *H. annosum* damage was found in more than 10% of the stands (up to 44.6% of the pine stands area was with the pathogen in roots of trees) (Sierota 1995).

In Poland, losses caused by *H. annosum* in middle-aged Scots pine stands established on former agricultural soils and farm abandoned land were as follows: stand density decreased from 0.9 to 0.6, current annual increment decreased by 50-60%, standing volume decreased by 27.8-69.5 m^3/ha, depending on intensity of fellings (Rykowski and Sierota 1984, Sierota 1997c).

Poland was the second country in the Europe (after UK), where as early as 1970 the Rishbeth's (1959, 1975) ideas to control the root pathogens were introduced and first practical experiments with *P. gigantea* on semi-economical scale were done (Sierota 1975). This fungus is a known natural component of coniferous forest ecosystems. In the Forest Research Institute in Warsaw, the original method of production and practical application of "Pg-IBL®" preparation with the competitor was developed in 1970s and 1980s and is still being improved (Rykowski and Sierota 1977, Pratt et al. 2000). The preparation "PgIBL®", consisting of viable mycelium of *P. gigantea* grown on sterilized beech (*Fagus*) sawdust, is used to prevent the colonization of stump surfaces by *H. annosum* and the development of the pathogen in whole root system. *P. gigantea* in "Pg-IBL®" decomposes wood of pine roots rapidly - up to 52% of the dry mass of lateral roots in six months (Sierota 1997a, 1998), and in addition reproduces rapidly in the stand.

The reduction in *H. annosum* spread throughout coniferous stands and a significant decrease in tree mortality resulting in an increase of crop production are the main economic benefits of using *P. gigantea* (Sierota 1998). In State Forest, artificial inoculation of stumps with *P. gigantea* in PgIBL®-like preparation in first-rotation Scots pine stands, established on former agricultural land, has been obligatory since 1984. By 1992, PgIBL was used on approximately 18.2 million freshly cut stumps; in 1992-1998 the preparation was used satisfactorily in an area of 360 200 hectares of thickets and stands over 20 years old, and in 1999, on 84 000 hectares (Fig. 1).

The efficiency of spring and fall treatments is even 100% (measured next year by presence of fruitbodies and mycelium in stump roots) as long as stumps are sufficiently handled (tapping, covering with litter, etc.). The latter increases labour costs by approximately 10%; however, these costs are counterbalanced both by ecological and economical advantages.

The success of the biological method of prevention and control of root rot in Scots pine stands in Poland (Sierota 1984, 1997b, 1998) results in: a decrease in primary infection risk from the stump surface side: a decrease in primary infection risk from the root side: greatly reduced production of *H. annosum* fruitbodies; rapid and effective decay of the root system; enrichment of the developing forest site on former agricultural land with saprotrophic Basidiomycetes (many strains of *P. gigantea*) which play an important role in the energy balance of the forest ecosystem.

Using PgIBL®-like preparation during routine protection treatments of pine stands on former agricultural land lessens tree mortality by 43% and increase the production of thickwood volume by 19 m^3/ha, if compared with stands where protection treatments are not carried out. At the scale of PgIBL use amounting in Poland to about 55 000 hectares per year in last decade, avoiding the loss could be estimated at the level of about US$20 million yearly.

Forest plantations on former agricultural soils are artificial ecosystems. Diseases and other harmful effects that occur in these plantations reflect natural adaptation. These adaptive changes, however, do not match the goals of traditional forest management and do not introduce advantageous effects from the anthropogenic point of view. The creation of stable and pest-resistant forests on former agricultural land is not possible without properly-directed management efforts at each and every stage of stand development. Biological control of root rot in threatened stands, particularly using *P. gigantea* and similar acting preparations, according to Rishbeth's ideas, plays still a fundamental role in this concept (Rykowski 1990, Sierota 1995).

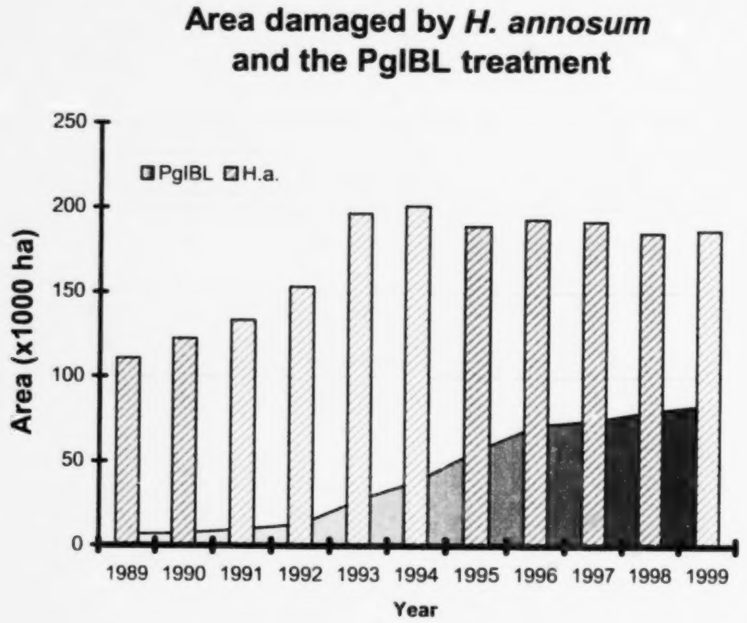

Figure 1. Area damaged by *H. annosum* and treated with *P. gigantea* formula PgIBL.

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STUMP INOCULATION WITH *PLEUROTUS OSTREATUS* (JACQ.: FR.) P. KUMMER

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SUMMARY

Initial field experiment was made in order to test the wood decomposing fungus *Pleurotus ostreatus* for biological control of *Armillaria*.

Field experiment with application of biological preparation of *P. ostreatus* (in form of beech sawdust with growing mycelium) on deciduous tree stumps was performed in two mixed beech-oak-pine (Norway spruce) stands. Sixteen month after treatment beech, oak and birch stumps (one of each species) were rooted out and cut into 10 cm sections. Samples of sections' wood were then used (pieces of wood incubation on malt-agar medium) to check the presence of *P. ostreatus* mycelium. The deepest colonization of stump's wood with *P. ostreatus* was present 20 cm from the stump surface, where the preparation was initially applied. Oak wood was much less colonized – *P. ostreatus* mycelium was found about 5 cm deep from the stump surface.

Keywords: stump inoculation, biological preparation of *Pleurotus ostreatus*

INTRODUCTION

Armillaria root disease causes very serious economic problems in the forests of Poland. Damages were registered in 2000 in deciduous and coniferous stands of all age classes over an area of more than 133 000 hectares (Sierota et al. 2001).

There are various approaches and techniques and avoidance of this disease in forests and orchards (Hagle and Shaw 1991, Fox 2000). Biological control methods seem to be very promising in the forests (Hagle and Shaw 1991, Raziq 2000).

Artificial inoculation of stump surface using saprotroph organisms is the one of the ways to reduce food base (stumps) that is necessary to the life cycle of *Armillaria*.

The aim of this work was to test a biological preparation containing the wood decomposing fungus *Pleurotus ostreatus* (Jacq.: Fr.) P. Kummer for application on deciduous tree stumps oak (*Quercus spp.*), beech (*Fagus silvatica*) and birch (*Betulus pubescens*) in the stands threatened by *Armillaria*. Growth of *P. ostreatus* in the wood of stumps was observed.

MATERIAL AND METHODS

The biological preparation of *P. ostreatus* is composed of beech sawdust with growing mycelium of *P. ostreatus*. Pure culture of *P. ostreatus* obtained from fruit body growing on dead birch (Krasiejow Forest District) was inoculated on sterilized sawdust medium and incubated for 2 months.

Testing of the biological preparation with *P. ostreatus* was done in stumps of deciduous species in the forest. In April 1999, two field plots were established in Strzebielino Forest District in stands with domination of beech (*Fagus silvatica*) (I plot: N 54°35′and E 18°05′ and II plot: N 54°30′ and E 18°01′). Trees were cut manually during thinning. The stump treatment was performed manually seven days after the cutting operation. Before treatment, a stump disc about 3-5 cm thick was cut off. One or two parallel or cross incisions were made on the stump. The substrate overgrown with mycelium and water was put on the stump in a layer about 0.5 cm thick. The woody disc (cut earlier) had been placed on the stump surface with prepared pulp and it was fixed with a nail (Rykowski and Sierota 1982).

Twenty beech, 15 oak and 10 birch stumps were inoculated. Sixteen months after treatment, three stumps (beech, oak and birch) were analyzed macroscopically. The soil around the stumps was removed, butt swellings were uncovered, bark was removed and colonization of wood by *P. ostreatus* was assessed. The occurrence of other fungi species, both pathogen and saprotroph, was searched out. Finally, stumps were rooted out and taken for further laboratory analysis.

In the laboratory, the stumps were washed under current water and cut into 10 cm sections. A macroscopic analysis of the condition of wood in collected stumps was made. Distinctions were made for: 1) wood colonized *by P. ostreatus*; 2) wood colonized by other fungi species; and 3) healthy wood, of proper structure and colour, not colonized by fungi. Then, after disinfecting the stump surface with ethanol, pieces of wood from 45 places (from upper butt surface, butt swellings, and bottom surface of stump) were taken out. From each place, eight small pieces of wood were taken. In total, 360 small pieces of wood were transferred on malt agar in Petri dishes.

Pure cultures of obtained fungus were identified:

a) by comparing with standard pure cultures of *P. ostreatus* from the Museum of Cultures;
b) with mating test (Korhonen 1978) to identify *Armillaria*;
c) with mycological keys (Barnett 1955, Domsch and Gams 1970).

RESULTS

From the total amount of 360 pieces of wood from investigated stumps, pure fungi cultures were obtained from 212 ones. From the remaining 148 pieces of wood, no mycelia developed (Table 1).

P. ostreatus was easily identified by comparing with pure standard cultures of this fungus and checking the occurrence of clamp connections. *P. ostreatus* was isolated from surfaces of beech and birch stumps in 100% and from oak in about 30%.

P. ostreatus was isolated from butt swellings of beech, birch and oak stumps in about 30%.

P. ostreatus was isolated from bottom surface of beech stump in about 30% and of birch stump in about 10%.

The deepest colonization of stump's wood with *P. ostreatus* was present 20 cm from the stump surface, where the preparation was initially applied (beech and birch stumps). Oak wood was much less colonized – *P. ostreatus* mycelium was found about 5 cm deep from the stump surface.

Table 1. Fungi isolated from wood fragments of beech, birch and oak stumps.

Fungus species	Stumps inoculated with biological preparation of *Pleurotus ostreatus*			Total
	beech	birch	oak	
	No	No	No	No
1. Wood fragments taken from surfaces of stumps				
Pleurotus. Ostreatus	24	32	8	64
Number of wood fragments unsettled by fungi	-	-	16	16
Number of wood fragments from which isolations were performed	24	32	24	80
2. Wood fragments taken from butt swellings				
Armillaria ostoyae	16	-	-	16
Graphium spp.	-	-	8	8
Pleurotus ostreatus	8	8	8	24
Unsporificating cultures	-	8	8	16
Number of wood fragments unsettled by fungi	-	8	-	8
Number of wood fragments from which isolations were performed	24	24	24	72
3. Wood fragments taken from bottom surfaces of stumps				
Pleurotus ostreatus	24	8	-	32
Trichoderma polysporum	-	-	8	8
Acremonium spp.	-	8	-	8
Zygorhynchus moelleri	-	4	-	4
Unsporificating cultures	8	16	8	32
Number of wood fragments unsettled by fungi	40	36	48	124
Number of wood fragments from which isolations have been performed	72	72	64	208

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COLONISATION AND DEGRADATION OF SITKA SPRUCE SAPWOOD BY THE ROTSTOP STRAIN OF *PHLEBIOPSIS GIGANTEA*

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SUMMARY

Degradation of living Sitka spruce (*Picea sitchensis*) sapwood by the Rotstop strain of *Phlebiopsis gigantea* was examined in samples removed from trees inoculated 18 months previously using colonised Scots pine dowels. Only *P. gigantea* was re-isolated from the inoculation points; sites inoculated with control (sterile) dowels became colonised by *Stereum* spp. Hyphae were present in earlywood tracheids both above and below the points of inoculation, with significantly greater numbers present above the inoculation point than at the point of inoculation, or below. Scanning electron microscopy revealed the penetration of hyphae through pits in the tracheid walls. Coalescence of penetration points through pits formed large boreholes. These structural alterations were not found in control inoculations. Observations of thin sections using transmission electron microscopy revealed symptoms associated with both simultaneous rot and soft rot in sapwood colonised by *P. gigantea*. In most observations, hyphae present within the tracheid lumen caused a gradual erosion of the cell wall from the S3 layers inwards through to S2 and S1/middle lamella, with decay occurring in the tissues immediately adjacent to the hyphae. Erosion lead to penetration of the cell wall. Penetration through pits and the formation of large boreholes accounted for the rapid spread of the fungal hyphae and the formation of the majority of cavities within the cell wall. It was concluded that in Sitka spruce wood the biocontrol agent Rotstop will compete directly with the pathogen (*Heterobasidion annosum*) for the woody resource.

Keywords: Sitka spruce, Rotstop, *Heterobasidion annosum*, biological control, cell wall degradation

INTRODUCTION

Rotstop is a commercial formulation of *Phlebiopsis gigantea* prepared for the biocontrol of *Heterobasidion* spp. on Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) (Korhonen et al. 1994). Although trials have been carried out on its ability to prevent *H. annosum* and *Heterobasidion parviporum* infecting *P. abies*, particularly in Finland and Scandinavia (Korhonen et al. 1994), similar studies on other species of spruce of commercial importance in EU countries have only recently started (Soutrenon et al. 1998). A trial was designed to test the ability of Rotstop to colonise and degrade living *Picea sitchensis*, as a precautionary measure before this strain of *P. gigantea* is released as a stump treatment against *H. annosum* in Britain. *P. sitchensis* is of great commercial significance on the north western maritime fringes of Europe, accounting for 40% of the timber produced in the UK (Crowther et al. 1991). However, *P. sitchensis* is also very susceptible to decay by *H. annosum*, particularly on mineral soils (Redfern and Ward 1998). Current controls are almost entirely based on the prophylactic use on fresh stumps of urea or borates (Redfern and Ward 1998; Pratt 2000), with good success rates. The availability of an effective, self-perpetuating biocontrol agent (BCA) would be of great advantage in improving current preventative practices. It is important, however, to ensure that a decay-causing fungus introduced as a BCA does not pose a phytosanitary risk to the host trees. This paper reports the colonisation and degradation patterns caused by the Rotstop strain of *P. gigantea* in stems of living *P. sitchensis*.

MATERIALS AND METHODS

Inoculations and sampling: Freshly-cut 20 mm diam. Scots pine sapwood dowels, approximately 20mm long, were sterilised by autoclaving at 105 kPa for 60 minutes, then inoculated with *P. gigantea* cultures and incubated at 28°C for 1 month. Control dowels were similarly sterilised but incubated on malt extract agar alone. On 29 September 1998, two 80 mm diameter bark pieces were removed at breast height at points 90° - 180° from each other in cardinal positions selected at random on each of ten standing 43-yr-old Sitka spruce near Copenhagen (55°N 12°E). In each tree, one colonised or one sterile dowel was inserted into a 20 mm diam. hole drilled in the centre of each decorticated zone. Trees were felled 17 months later, on the 6 March 2000, dissected and samples stored at approximately 4°C until required. From one of the ten trees, sapwood samples 30 x 20 x 7.5 mm were removed, from around each dowel, and from 200 mm above and below the dowel insertion point.

Re-isolation of *Phlebiopsis gigantea* from sapwood blocks: Samples of tissue (approx. 5 x 1 mm were removed from the sapwood blocks and plated onto 2% malt extract agar containing antibiotics (20,000 units penicillin, 200mg streptomycin). Following incubation at 22°C for 7-10 days, cultures were examined for oidiospores, characteristic of *P. gigantea*.

Colonisation of sapwood: From each sapwood sample, a block 15 x 10 x 0.75 mm was cut and sectioned using a sledge microtome. Sections (25-30 μm) were stained in toluidine blue and examined under the microscope. Colonisation was quantified by selecting a point within the section at random, disregarding any position <5 mm from the top or bottom of the section, and counting the number of tracheids containing hyphae. Three positions were examined for each section, and five replicate sections per sample block.

Scanning electron microscopy (SEM): After removal of thick sections , sample blocks were washed in distilled water and fixed for 24hrs in 1.6 % paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.4. Tissue was washed in 0.1M sodium phosphate buffer, pH7.4, (4x15mins), dehydrated in an ethanol series and critical point dried with liquid carbon dioxide, using a Polaron E3100 Critical Point Dryer. Dried samples were mounted on aluminium stubs and sputter coated with gold in an Emitech K550 sputter coater. Coated samples were examined using a Cambridge Instruments S600 scanning electron microscope at an operating voltage of 7.5kV.

Transmission electron microscopy (TEM): A further set of samples (0.5 x 1 x 1 mm) were cut, rinsed in distilled water and fixed as described above. Tissue was washed in 0.1M sodium phosphate buffer, pH 7.4, (4x15mins), secondary fixed in 1% osmium tetroxide (aq) for 2hrs, and dehydrated in an ethanol series. Samples were infiltrated with Taab Embedding Resin (medium grade), embedded and polymerised at 60°C for 24 hrs. Transverse sections (100-120 nm) were cut using a Reichert Jung Ultracut Ultramicrotome, stained with uranyl acetate and lead citrate and examined with a Philips 301 TEM at 80kV.

RESULTS

Isolation of fungi from sample blocks: *P. gigantea* was isolated from this tree from positions around the point inoculated with *P.gigantea*, and from the blocks removed from 200 mm above and below it, but not from the side of the tree inoculated with a sterile dowel. Segmentation of the hyphae *in planta* into oidiospores confirmed the identity of *P. gigantea* in samples from the inoculation point. Fungi isolated from around the control inoculation point included *Penicillium* sp. and a *Stereum* sp.

Colonisation of tracheids by fungi: Hyphae were observed in each of the six sample blocks (Fig. 1A), though the extent to which the wood was colonised differed between blocks. Hyphal growth was greater in the earlywood tracheids than in latewood. Numbers of tracheids containing hyphae were significantly greater ($p = 0.032$) in samples near to the *P. gigantea* inoculation (783) point than in controls (277). Hyphal growth was significantly greater above *P.gigantea* inoculation points than below (406 and 148 respectively, $p < 0.001$).

Electron microscopy: Using SEM, hyphae of *P. gigantea* were observed penetrating pits in the tracheids (Fig. 1B). Here, degradation of cell walls around the pits led to the formation of large boreholes, some of which coalesced (Fig. 1C). Similarly large boreholes were not present in the sample blocks removed from the part of the tree inoculated with sterile dowels, although hyphae were not uncommon within tracheids (Fig. 1D).

When examined under TEM, no cell wall degradation was identified in sections from around the sterile dowel inoculation. However, hyphae were observed causing localised degradation on the surface of the S3 layer (Fig. 2A) in sections from *P. gigantea* inoculated positions. Here, degradation in the immediate vicinity of the hyphae lead to the formation of lysis zones, which eventually united causing a thinning of the cell wall (Fig. 2B). The S3 layer was degraded first, followed by the remaining layers of the secondary cell wall (Fig. 2C). Eventually, all layers of the cell wall were degraded, with the cell corners remaining (Fig. 2D). In some sections, cavities within the S2 layer containing hyphae were noted (Fig. 2E).

DISCUSSION

Only a *Penicillium* sp. and a *Stereum* sp. were found in the wood surrounding the control inoculation, and little degradation occurred over the 17 month experimental period. In contrast, *P. gigantea* was abundant in tracheids near to the inoculation point after this time. *P. gigantea* colonises xylem in pine and Norway spruce stumps rapidly after their inoculation (Meredith 1960; Korhonen, et al. 1994), a factor which undoubtedly contributes to the ability of this species to compete with *Heterobasidion* spp. in stumps infected by both organisms.

P. gigantea hyphae appeared to spread rapidly between *P. sitchensis* tracheids through the penetration of pits and by formation of boreholes, as described previously by Liese (1970) for other simultaneous decay fungi. Large boreholes, with diameters many times greater than the hyphae within them resulted from decay caused by hyphae passing through pits. In some cases boreholes coalesced and thus increased the apparent rate of cell wall degradation. The formation of boreholes and subsequent T- or L-branching of hyphae within the S2 layer were probably responsible for the formation of cavities within the cell wall. (Cf. Daniel et al. 1992).

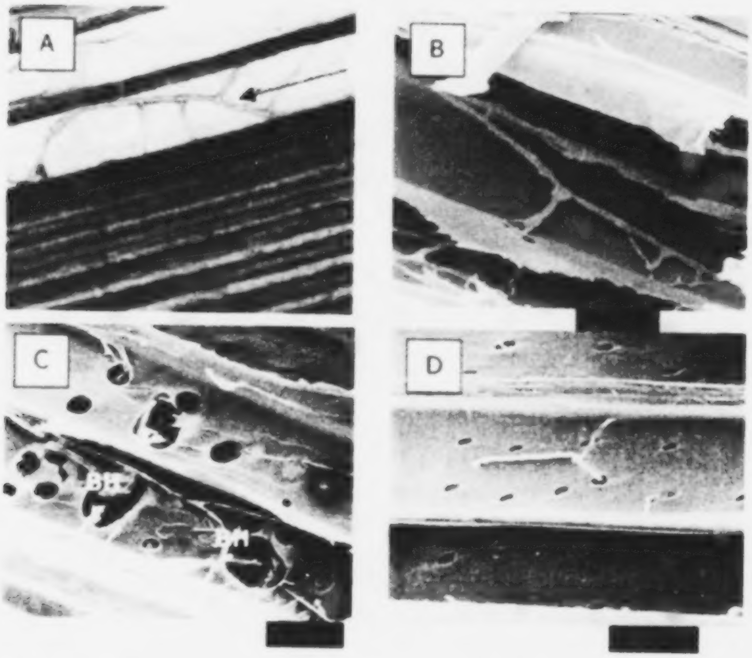

Figure 1. (A) Hyphae of *P. gigantea* in earlywood tracheids; (B) SEM micrograph: *P. gigantea* hyphal branching; branch is passing through pit in cell wall; (C) Penetration of pits by hyphae has resulted in formation of large boreholes (BH) in the cell wall; (D) A tracheid from sample block 5 (inoculated with sterile dowel). Note the size and appearance of the pits in wood not colonised by *P. gigantea*. (B-D, Bar = 20 μm).

Within tracheids, degradation of the S3 layer occurred initially, followed by the successive decay of the S2 and S1 layers and the compound middle lamellae, with the cell corners degraded last. This pattern suggests that degradation of cellulose and lignin occurred simultaneously and thus the term "simultaneous rot" may be assigned to *P. gigantea* in the conditions of this experiment (Blanchette 1991). This conclusion was further supported by the observation that cell wall degradation occurred in the immediate vicinity of hyphae (Otjen and Blanchette 1986; Liese 1970). The presence of erosion furrows (Schwarze et al. 1999; Liese 1970) was also noted where fungal hyphae on the S3 layer had grown deeper into the cell wall.

Although reported only rarely in coniferous wood, simultaneous rot in conifers has been shown to occur under certain conditions (Schwarze et al. 1999). In the context of this work, it is relevant to note that *H. annosum* has been observed to cause simultaneous lignin and cellulose degradation under laboratory conditions in spruce wood (Meier 1955). It now seems likely that *P. gigantea* has the ability to cause a selective delignification within the same substrate. The substrate cell types and other micro environmental conditions affect degradation patterns (Blanchette 1991) but the exact mechanism by which selectivity for lignin occurs is not fully understood (Maijala 2000). The view that white rot fungi can cause both selective delignification and simultaneous rot has been suggested previously (Otjen and Blanchette 1986; Schwarze et al. 1995).

In addition to the simultaneous rot discussed above, the presence of *P. gigantea* hyphae within cavities formed in the S2 layer of the cell wall also suggests a soft rot decay mode (Schwarze et al. 1995). Similar cavities have previously been reported in *Pinus sylvestris* decayed by *Oudemansiella mucida* (Daniel et al. 1992). Co-occurrence of simultaneous rot and soft rot was also described in decay of *Platanus* x *hispanica* by *Inonotus hispidus* (Schwarze et al. 1995). It was suggested that each mode of decay arose in regions of the wood with different micro-environmental conditions.

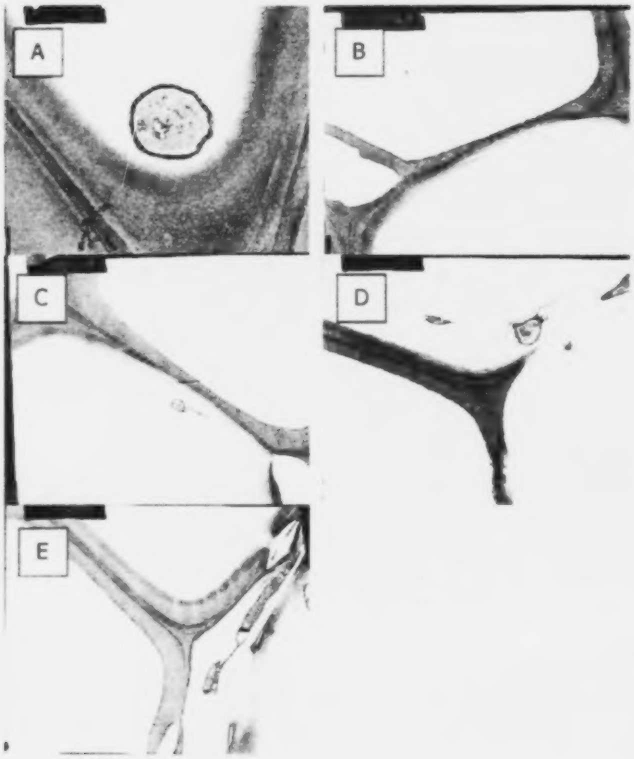

Figure 2. (A) *P. gigantea* hypha in lumen causing localised degradation of the S3 layer (Bar = 1.25 µm). (B) As decay progressed, neighbouring lysis zones united resulting in a thinning of the S3 and S2 layers (Bar = 7.5 µm). (C) Extensive degradation of S3, S2 and S1 layers. Hypha is visible on cell wall (Bar = 7.5 µm). (D) Decay immediately around a hypha has resulted in penetration of cell wall, possibly due to a large borehole being formed. Middle lamella at cell corner remains virtually intact (Bar = 7.5 µm). (E) Hyphal growth within the S2 layer of the cell wall, indicative of soft rot (Bar = 7.5 µm).

This Rotstop trial was initiated in Denmark to determine the risk, in the UK, to a commercially-valuable exotic spruce from a non-indigenous strain of a saprotrophic fungus, other strains of which have been used in the UK as a BCA on pine stumps for many years (Pratt et al. 2000). Denmark was chosen since both the host and the BCA agent were established there. The work reported here needs to be considered alongside research into the DNA of Rotstop conducted in Scandinavia (Vainio and Hantula 2000) and in Britain (unpublished), and does not in itself provide any contra-indications for the release of Rotstop in the UK as a stump treatment against *H. annosum*. Indeed, the capacity of this strain of *P.gigantea* to colonise living Sitka spruce sapwood by utlising those components required for the growth of the pathogen (Korhonen and Stenlid 1998) would suggest that this or similar strains of *P.gigantea* would be suitable candidates for the biocontrol of *H.annosum* on stumps of Sitka spruce.

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SIMULATED STUMP TREATMENT EXPERIMENTS FOR MONITORING THE EFFICACY OF *PHLEBIOPSIS GIGANTEA* AGAINST *HETEROBASIDION*

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SUMMARY

The efficacy of the biological stump treatment agent Rotstop® against *Heterobasidion* was checked by regularly simulating the stump treatments in log pieces. The pieces were cut from the basal part of Norway spruce and Scots pine stems; pieces were 20 (-30) cm long and 14-18 cm in diameter. One half of the upper surface was treated with *P. gigantea* suspension and, ½ - 1 h later, the whole surface was sprayed with a conidial suspension of *Heterobasidion*. The pieces were kept standing on moist sand in the open air in half-shadow (some experiments were made in the greenhouse). After ca. 6 weeks, sample discs were cut from the log pieces, incubated for 5-7 days in plastic bags, and then checked for the occurrence of *Heterobasidion* conidiophores. The efficacy of the treatment was calculated by comparing the areas occupied by *Heterobasidion* on the two halves of each piece of log. These experiments have been carried out every year since the introduction of Rotstop in 1993. The results indicate that the *P. gigantea* strain of Rotstop has retained its efficacy over the course of ten years. Compared with other *P. gigantea* strains originating from different parts of Europe, the Rotstop strain has proved to be one of the most effective ones, but it is not essentially better than other good strains. For spruce wood the preparation seems to be slightly more effective against *H. annosum s. str.* (P type) than against *H. parviporum* (S type). Mixtures consisting of two to four *P. gigantea* strains were generally at least as effective as the individual strains. The efficacy of the treatment for protecting spruce wood seems to be almost directly proportional to the area covered by the protectant on the cut surface, but for pine wood the coverage is not so critical for good efficacy.

Keywords: *Heterobasidion annosum*, *H. parviporum*, *Phlebiopsis gigantea*, stump treatment, biological control

INTRODUCTION

Stump treatment with the *Phlebiopsis gigantea* (Fr.) Jül. preparation Rotstop® started in Finland in 1993. Since then the Finnish Forest Research Institute has carried out experiments every year in order to control the efficacy of the preparation. Because stump treatment experiments in the forest are relatively laborious, the stump treatment was simulated in pieces of spruce or pine logs. The main results of these experiments are presented shortly in this paper.

MATERIAL AND METHODS

Norway spruce or Scots pine stems with a stump diameter of about 18 cm, and with as little tapering and as few branches as possible, were selected for the experiments. The stem was cut into 125 cm long sections and carried to the laboratory. On the same or following day they were cut into 20 cm-long pieces (occasionally 30 cm). The maximum number of pieces from one stem was 30. After cutting, the upper surface was divided into two symmetrical halves by means of a marker line. One half was covered, and the other half was sprayed with the protecting agent. About ½ - 1 h later both halves were sprayed with a conidial suspension of *Heterobasidion* (ca. 50-100 spores/cm^2). A mixture of conidia taken from 3-5 heterokaryons was usually used. The number of living spores in all the treatment suspensions was determined before and after treatment. The treated log pieces were kept standing on moist sand for 5-6 weeks outdoors in half shadow. They were watered occasionally during dry periods. A number of the first experiments were carried out in the greenhouse.

At the end of the experiment, one or more sample discs were cut from the logs, washed and incubated in plastic bags for 5-7 days. A transparency with a square centimetre grid was fixed on the disc, and the area occupied by *Heterobasidion* on the disc surface was determined under a dissection microscope. The area occupied by *P. gigantea* was also approximately determined on the basis of the characteristic orange-brown colour on the disc surface. At least two depths were investigated: usually down to a depth of 3 cm and 8 cm from the treated surface. The efficacy of the treatment was calculated by comparing the area occupied by *Heterobasidion* on both halves of the disc. The number of repetitions was usually 9 (three stems, three repetitions in each stem).

RESULTS

About 20 log experiments have been carried out since 1992. The experiments have concentrated on the following aspects:

Colonisation of spruce and pine wood by *P. gigantea* and *Heterobasidion*. *P. gigantea* colonises pine sapwood much more effectively than spruce wood. In spite of the relatively low colonisation in spruce, the efficacy of *P. gigantea* against *Heterobasidion* is usually high, obviously because these two fungi are competing for the same part of the wood. Both *H. parviporum* Niemelä & Korhonen and *H. annosum* (Fr.) Bref. *s.str.* are able to colonise spruce and pine wood, but the former species colonises more effectively spruce and the latter species pine (Fig. 1).

Efficacy of Rotstop against different species of *Heterobasidion*. In the case of spruce wood, Rotstop more effectively controls infection by *H. annosum s.str.* than by *H. parviporum*. However, the difference is not great (Fig. 2). Both homo- and heterokaryons are controlled to the same extent.

Number of spores in the treatment suspension. In order to guarantee good efficacy of *P. gigantea* on spruce, the number of living spores in the treatment suspension should be 5 million per litre or more. However, the efficacy of the Rotstop strain decreases relatively slowly down to a concentration of 1 million spores/l (Korhonen et al. 1994).

Relationship between treatment coverage and efficacy. On spruce, the efficacy is almost directly proportional to the size of the area treated on the stump surface (Fig. 3). In pine wood, *P. gigantea* spreads more easily in a horizontal direction, and the efficacy percentage is greater than the covering percentage.

Effective strains. In most experiments in which the Rotstop strain has been compared with other isolates originating from Europe, it has proved to be one of the best. So far no other isolate has proved to be distinctly superior in repeated experiments. On the other hand, it should not be difficult to find isolates from nature that have a similar level of efficacy. In some experiments the efficacy of Rotstop has not been good, but in most of these cases the number of spores in the preparation was low (1-3 mill. spores/g).

Isolate mixtures. The use of mixtures containing 2-5 different *P. gigantea* genotypes appears to be possible without any reduction in efficacy (as a result of intra-species competition). On the other hand, mixtures do not significantly increase the efficacy (Fig. 4).

CONCLUSION

During almost 10 years of use, the experiences gained with Rotstop have generally been good (e.g. Korhonen et al. 1994, Thor and Stenlid 1998, Soutrenon et al. 2000). The results of the experiments described above indicate that the preparation has retained its efficacy. However, there have occasionally been problems in keeping the spore concentration at a sufficiently high level (above 5 mill. spores/g) in every lot. Changing the strain may be desirable in the near future, and it will be relatively easy to find strains with comparable efficacy.

In the year 2000, about 9000 ha of thinnings and 3 000 ha of final cuttings, mostly spruce, were treated in Finland. Practically all of this area was treated with Rotstop, the rest with 30% urea solution. Continuous quality control of the stump treatments is needed, especially because the state presently subsidises this activity by reimbursing a private forest owner for part of the costs of stump treatment. Besides the quality of the protectant, control should include the work performance, total long-term efficacy, and the effects of the treatment in forest ecosystems.

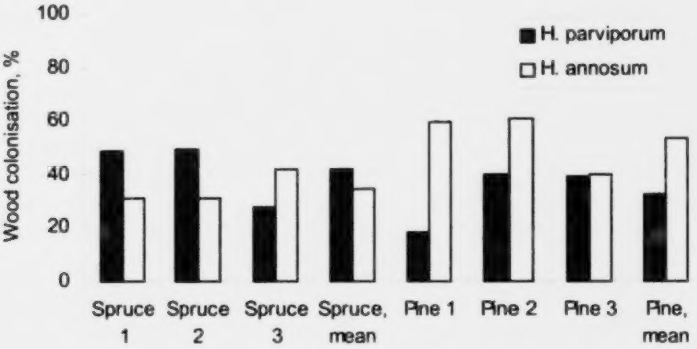

Figure 1. Wood colonised by some Finnish strains of *H. parviporum* and *H. annosum s.str.* in pieces of spruce and pine logs six weeks after inoculation. The logs were cut from three Norway spruce and three Scots pine individuals. Each column of individual trees (1-3) represents the mean colonisation of eight heterokaryons of *Heterobasidion*. The picture illustrates the situation at a depth of 3 cm from the treated surface. The experiment was made in the greenhouse; temperature varied between 15 and 25°C.

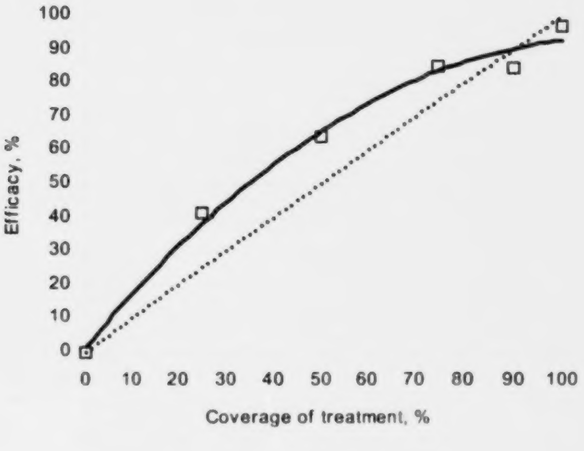

Figure 2. Efficacy of Rotstop against homo- and heterokaryotic isolates of *H. parviporum* and *H. annosum s.str.* and their mixtures in pieces of spruce logs. All the isolates originated from Finland. Results from depths of 3 and 8 cm from the treated surface are presented. The experiment was made in the greenhouse.

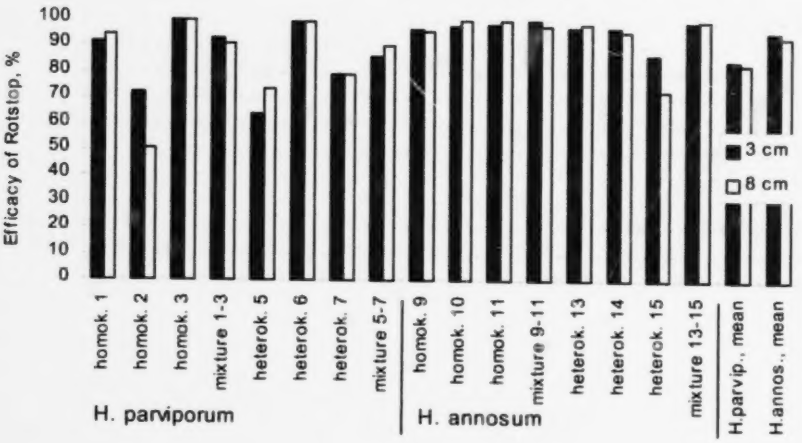

Figure 3. The relationship between the coverage of the Rotstop treatment and the efficacy against *H. parviporum* in pieces of spruce logs. The dashed line shows the linear correlation. The experiment was made outdoors in June – July 1997.

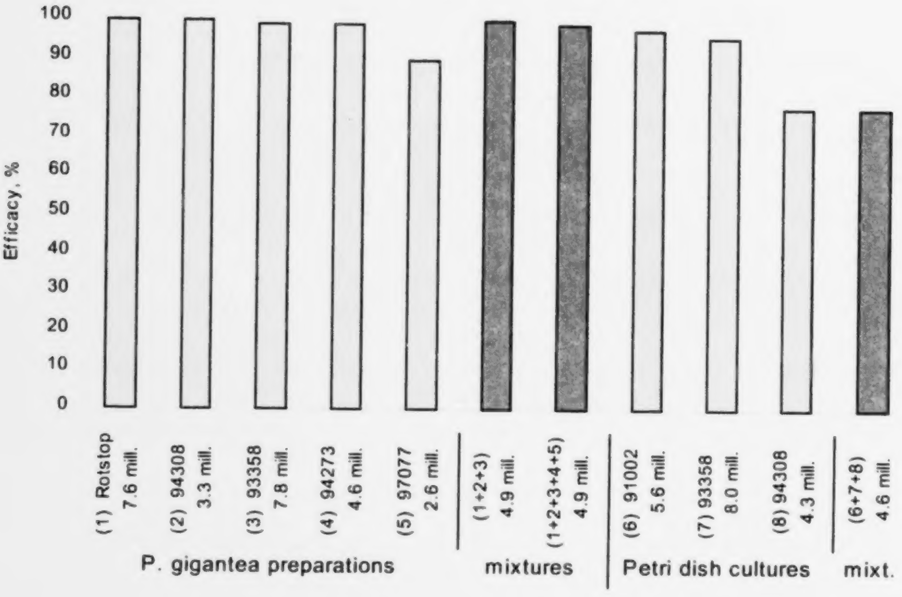

Figure 4. Efficacy of five *P. gigantea* preparations (made by Kemira Agro) and their mixtures against *H. parviporum* in pieces of spruce logs. In addition to the preparations, petri dish cultures of three isolates were tested for comparison. Spore concentration per litre of treatment suspension is indicated. The experiment was made outdoors in July – August 1998.

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PRELIMINARY RESULTS USING BIOLOGICAL CONTROL AGAINST *HETEROBASIDION ANNOSUM* ON SILVER FIR IN SOUTHERN ITALY

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SUMMARY

Preliminary results of biological control treatments against *Heterobasidion annosum* (group F), carried out in a 65-year-old Silver fir plantation in southern Italy are reported. In particular, *Hypholoma fasciculare*, *Ptychogaster rubescens*, *Phlebiopsis gigantea* (Rotstop®) and *Trichoderma viride* were inoculated as test fungi together with the pathogen, aiming at assessing their ability to colonise Silver fir wood despite *H. annosum* occurrence.

Inoculations were carried out on 30 stumps obtained after felling apparently healthy trees. Either woody blocks or conidial suspensions were used as inocula, and sterile wood or water, as a control. Results were collected over one year after treatments at six months intervals: differences in percentages among fungal isolations from woody samples were assessed and statistically analysed.

All treatments did not significantly reduce the amount of *H. annosum* isolations compared to controls. *P. gigantea* was the most isolated test fungus, *H. fasciculare* the least and *P. rubescens* was mostly recorded at the first survey. These experiments confirmed *P. gigantea* effectiveness in colonising Silver fir wood, despite the presence of the pathogen. *H. fasciculare* and *P. rubescens* need to be further surveyed for being considered as possible antagonists, while *T. viride* revealed to be unable to control *H. annosum* in wood.

Keywords: *Heterobasidion annosum*, Silver fir, antagonistic fungi, biological control

INTRODUCTION

Heterobasidion annosum (Fr.) Bref. is one of the most important root and butt rot agent fungi of forest trees, especially conifers, in the world (Woodward et al. 1998).

The most affected conifer species in southern Italy is Silver fir (*Abies alba* Mill.), particularly where site conditions are less favourable due to drought and/or saprotroph microorganisms deficiency of the soil as a consequence of previous pasture or agricultural land use (Capretti 1998; Luisi and Sicoli 1993).

In northern and central Italy, experiments using biological control against *Heterobasidion annosum* (Fr.) Bref. have suggested that this may be an effective method of stump treatment (Capretti and Mugnai 1989; Nicolotti et al. 1999). However, in southern Italy, there have so far been few studies using antagonistic fungi as biological control agents against *H. annosum*. Experiments were initiated in a Silver fir plantation already affected by *H. annosum*. In particular, the ability of some wood-degrading fungi to colonise Silver fir wood was tested in order to assess their potential as a possible tools against the pathogen.

MATERIALS AND METHODS

The experimental site was a 65-year-old Silver fir plantation (ca. 45 ha in area), ca. 800 m a.s.l. in the Foresta Umbra, province of Foggia, southern Italy. Here, attacks by *H. annosum* group F (= *H. abietinum* Niem. & Korh.) (Capretti et al. 1998) have been observed over the last 20 years (Capretti and Moriondo 1983).

In this stand, 30 apparently healthy trees, 20-30 cm of diameter at 1.30 m dbh, were selected at random and cut at about 55 cm above the collar. A 5 cm thick disk was removed from the top of each stump immediately prior to treatment application. This disk was later fixed with nails as a lid over the inoculum, to cover the top of the stump once the treatments had been applied (Nicolotti et al. 1999).

1. Treatment A (n = 20 trees)

One isolate of *Hypholoma fasciculare, Ptychogaster rubescens* and *Phlebiopsis gigantea* (Table 1) were chosen as test fungi. Inocula consisted of 1.0 x 1.5 x 6.0 cm Silver fir wood blocks previously autoclaved at 121°C for 40 min and then colonised by the fungus for 3 months at 22±1°C in the dark, in 10 cm Petri dishes filled with Malt Extract Agar (MEA) 2%. Inoculations were carried out in April 2000, immediately after tree felling, the antagonist was introduced on the west side of the stump and *H. annosum* on the east one. Inoculation points were 15 cm apart. Colonised wood blocks were carefully placed into pre-drilled holes in the surface of the stump.

Each antagonist/*H. annosum* combination was replicated five times, and five stumps were subjected to sterile wood/*H. annosum* inoculation as a control.

2. Treatment B (n = 10 trees)

The test fungus was an isolate of *Trichoderma viride* Pers. ex Gray from a Silver fir plantation located in Monte Vulture, province of Potenza, at about 100 km from the site of the experiment. Twenty ml of conidial suspensions (2.3×10^7 conidia/ml for *T. viride* and $2,4 \times 10^5$ conidia/ml for *H. annosum*) were used as inocula, obtained after growing the fungi in 9 cm Petri dishes for 2 weeks on MEA 2% at 22±1°C in the dark. Spore suspensions were sprayed onto each of five stumps. The *T. viride* suspension was applied first and the *H. annosum* immediately following. The remaining five stumps were used as a control. Here, the *T. viride* conidial suspension was replaced by 20 ml of sterile distilled water.

3. Assessment

Prior to removing samples from the inoculated logs, the 5 cm lid was removed and discarded and a further 5 cm thick section was cut from the top of the stump. This second section was taken in order to avoid the possible occurrence of newly established wood-inhabiting fungi other than those of interest. This procedure was repeated at each sampling date.

Results were collected 6 months (October 2000) and one year (April 2001) after inoculation. Ten samples (1 cm^3 sized) were taken from the exposed surface of each stump and placed in sterile Petri dishes, sealed and taken to the lab. Five fragments per sample were then plated onto thiabendazole-lactate amended MEA 2% (Guillaumin et al. 1994) and incubated at 22 ± 1°C in the dark for 3 weeks. Isolations were also made on a non-selective medium (water-agar) to detect colonies of *Trichoderma*. The percentage of sample fragments producing colonies of the test fungi was assessed.

Differences in the ability of test fungi to colonise Silver fir stumps and to affect the growth of *H. annosum* were assessed by means of Duncan's test (SAS Institute Inc., 1989).

RESULTS

All three potential wood-degrading fungal antagonists were isolated six months after inoculation and again six months later, although the percentage recovery varied among isolates and between sampling dates (Fig. 1):

- Colonisation by *P. rubescens* was significantly greater than the other two fungi 6 months after inoculation;
- After one year, there was no significant difference in colonisation between *P. rubescens* and *P. gigantea*;
- Colonisation by *H. fasciculare* (and *T. viride*, data not shown) did not differ from control values;
- *H. annosum* colonisation of the stumps was always higher than that of the test antagonists and there was no apparent effect of the test fungi upon *H. annosum*.

H. fasciculare, *P. rubescens* and *P. gigantea* mycelial fans were visible on top of the stumps after removal of the 5 cm lid. *T. viride* was only visible on the surface of the stump at the 6 month sampling, although it was readily isolated from wood samples after one year.

In general, sampled woody fragments producing *P. gigantea* and *P. rubescens* cultures appeared more scattered than those producing *H. fasciculare* colonies, while *H. annosum* appeared to be widespread and well-established on all treated stumps.

DISCUSSION AND CONCLUSION

The data collected after both surveys showed that Rotstop® (i.e. *P. gigantea*) was the most effective in colonising Silver fir wood among the tested fungi. It remains to be seen whether the ability of *P. gigantea* to compete with *H. annosum* is confirmed in future studies.

H. fasciculare grew well on Silver fir stump surfaces during autumn and winter, despite the presence of *H. annosum*, but seemed to have limited ability to grow into the wood of Silver fir as it was rarely isolated from the stump tissue.

P. rubescens is a subtropical wood-inhabiting fungus, conidial form of the basidiomycete *Punctularia atropurpurascens* (Bark. & Br.) Petch (Stalpers 1978). It was isolated from a dead Olive tree stump collected about 300 km south of the Silver fir stand. It successfully colonised treated Silver fir stumps, but its presence in the wood decreased remarkably after autumn and winter. *In vitro* tests on MEA 2% and on woody blocks revealed that this fungus does not grow at 5°C (data not shown). Its antagonistic capability against *H. annosum* may be more appropriately tested in more suitable climatic conditions, i.e. in Mediterranean pine stands.

Treatments with *T. viride* did not give good results so far, despite preliminary *in vitro* tests on MEA 2% were promising (unpublished).

This research has been carried out in a stand where planted Silver fir trees are expected to be gradually replaced by a natural mixed broadleaved wood. It would appear that *H. annosum* is well-established in the fir, perhaps even inside some of treated stumps, and its presence may speed up the natural replacement of the fir by the natural vegetation. However, further surveys on treated stumps will be continued in order to check and assess the quantitative establishment of the potential fungal antagonists, as well as the occurrence of *H. annosum*.

Table 1. Fungal isolates used in biological control experiments (treatment A) against *H. annosum* on Silver fir in southern Italy.

Species	Host	Site origin
Heterobasidion annosum (Fr.) Bref.	*Abies alba* Mill.	Foresta Umbra (Foggia)
Hypholoma fasciculare (Huds.: Fr.) Kummer	*Abies alba* Mill.	Foresta Umbra (Foggia)
Ptychogaster rubescens Boud.	*Olea europaea* L.	Torre S. Susanna (Brindisi)
Phlebiopsis gigantea (Fr.) Jülich (Rotstop®)	*Picea abies* (L.) Karst.	FINLAND

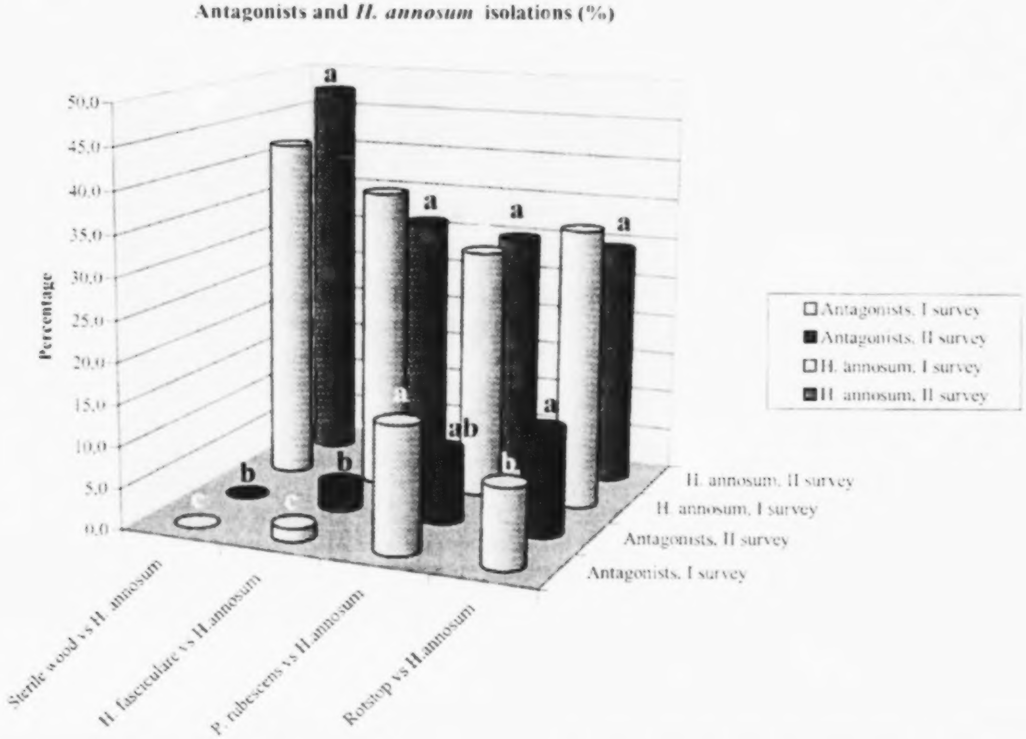

Figure 1. Mean percentages of antagonists and *H. annosum* isolations from treated stumps, six months (I survey) and one year (II survey) after inoculation (means referred to each survey and indicated by the same letter do not differ significantly at $p=0.05$).

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TESTING OF ROTSTOP ON SITKA SPRUCE, DOUGLAS-FIR AND LARCH

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SUMMARY

The ability of *P. gigantea* (Rotstop®) to colonize stem discs of Larch, Douglas-fir and Sitka spruce was tested in the laboratory. *P. gigantea* was able to grow on fresh discs cut from all three species, although in some cases, growth rates were less than on Norway spruce and Scots pine, which were used as reference trees. *P. gigantea* was able to prevent infection by *H. annosum* on all five species. The experiment indicates that Rotstop® may have potential for stump treatment on Larch, Douglas-fir and Sitka spruce. However, field trials are necessary to confirm these results.

Keywords: stump treatment, *Heterobasidion annosum*, *Phlebiopsis gigantea*

INTRODUCTION

In Denmark, all conifers are introduced, and Norway spruce is the dominant soft wood species. However, several other conifers are widely used, mainly Sitka spruce (*Picea sitchensis*), Douglas-fir (*Pseudotsuga menziesii*) and Larch (*Larix* x *eurolepis*). In addition, various pine species are planted on nutrient poor soils, including Scots pine (*Pinus sylvestris*). Most of the conifers used are considered susceptible to *H. annosum* rot and butt rot. Stump treatment with either urea or Rotstop is therefore recommended in thinnings and clear cuts.

Rotstop® is a biocide produced by Kemira Oy for stump treatment against *Heterobasidion annosum*. It consists of spores of *Phlebiopsis gigantea* based on an isolate found on Norway spruce (*Picea abies*) in Finland. In several trials in the Nordic countries, Rotstop® controlled *H. annosum* on Norway spruce (*P. abies*) and Scots pine (*Pinus sylvestris*) stumps. Only few trials on other conifer species have been reported.

The ability of Rotstop to compete with *H. annosum* on stump discs of Sitka spruce (*P. sitchensis*), Douglas-fir (*P. menziesii*) and Larch (*L.* x *eurolepis*) was tested in the laboratory as part of a bachelor thesis in Forestry at the Royal Veterinary and Agricultural University (Jacobsen 1998).

MATERIALS AND METHODS

One tree each of Larch, Sitka spruce, Douglas-fir, Norway spruce and Scots was felled, a 1 m length of stem was surface sterilized (70% ethanol) and cut into 2 cm thick discs on a band saw. Each disc was numbered and packed into a sterile plastic bag. The discs were randomly distributed to treatments, and the bottom disc was incubated to make sure that *H. annosum* was not already present. The average disc diameter was 12 cm. Norway spruce and Scots pine had no visible heartwood.

Each combination of tree species and treatment was represented by five discs. For inoculation with *P gigantea*, an aqueous suspension of working-strength Rotstop® (kindly donated by Kemira Oy) was made and applied by spraying at a rate equivalent to 200-300 spores/cm^2. A P type isolate of *H. annosum* from an infected Douglas-fir was grown on PDA to provide conidia for inoculations at a rate equivalent to 100-400 spores/cm^2.

Spore concentrations were determined by counting chamber (haemocytometer), and the viability of suspensions by plating onto PDA.

Treatments:

1. *P. gigantea* alone.
2. *H. annosum* alone.
3. First *P. gigantea* and then *H annosum* on top, both on whole disc.
4. Division of disc in six wedgeshaped sections, three of which were treated with *P. gigantea*, after which the whole disc was inoculated with *H. annosum*.

The discs were put in paper bags, wrapped in dampened newspaper and incubated in plastic bags for 10-17 days at 20°C (Table 1). Infection of *H. annosum* was confirmed by presence of conidiophores. Infection by *P. gigantea* was detected by the presence of orange staining and strands of typical mycelia. In addition, *P. gigantea* was identified by microscopic examination of hyphae (double clamps and oidia). The areas occupied by each fungus were outlined on the discs and calculated. Re-isolations from the interior of the discs were also made.

As most discs were totally covered with mycelium of either species, the estimated **growth rate** based on the abundance of the mycelium provided the means of illustrating the success of infection and thus the suitability of the substrate for supporting superficial fungal growth. The growth rate of aerial mycelium was scored in three categories:

1. **Slow (score 1)** = mycelium only visible at 40 x magnification.
2. **Medium (score 2)** = mycelium visible at 6 x magnification.
3. **High (score 3)** = mycelium visible to the naked eye.

Table 1. Number of days since inoculation until the fungi could be identified.

Species	Scots pine	Larch	Sitka spruce	Douglas-fir	Norway spruce
H. annosum	10	10	10	10	17 *
P. gigantea	10	10	14	17	17 *

* The long incubation of Norway spruce was due to heavy contamination by moulds. After the 10 days of wet incubation, the discs were left 7 days under drier conditions and were then easier to assess.

RESULTS

Both *H. annosum* and *P. gigantea* infected discs from the five tree species to the extent that both fungi became widely distributed on discs of all species. However, there were clearly differences in the appearance of the surface mycelium on different species, and this attribute has been used to evaluate the success of treatment.

P. gigantea prevented the growth of *H. annosum* mycelia on all five tree species. On all discs treated with *P. gigantea* there was no growth of *H. annosum* visible, except for three cases with very small colonies ($< 1cm^2$). Re-isolation from wood below these colonies yielded only *P. gigantea*.

On discs that were not treated with *P.gigantea*, mycelium and conidia of *H. annosum* became visible on all five tree species after 10 days' incubation *P. gigantea* surface mycelia was markedly less effusive on Douglas-fir and Sitka spruce compared to Scots pine and Larch. However, *P. gigantea* managed to colonize entire disc surfaces even where mycelial growth was not abundant and prevent the establishment of *H. annosum* (Fig. 1).

Mycelium of both fungi was more luxuriant on sapwood than on heartwood, and results are therefore shown separately for both these tissues (Fig. 1). On sapwood, *P. gigantea* grew best on Norway spruce and Larch, almost as well on Scots pine, slower on Douglas-fir, and least on Sitka spruce (Table 2 and Fig. 2). On heartwood there was no significant difference. *H. annosum* in sapwood grew best on Scots pine followed by Norway spruce, then Douglas-fir and Larch, and slowest on Sitka spruce. In heartwood, growth on Sitka spruce was better than on Douglas-fir and Larch.

On the sectored discs, *P. gigantea* had overgrown adjacent but untreated sectors on pine after 10 days, but not on larch, Sitka spruce, or Douglas-fir. However, re-isolations from the interior of untreated sectors of discs from these tree species only yielded *P. gigantea*. After another week, surface mycelium of *P. gigantea* appeared on large parts of the untreated sections.

There were problems with contamination by moulds of disc surfaces, especially on Norway spruce. However, colonies of *H. annosum* and *P. gigantea* could still be determined.

Table 2. Average mycelial abundance on sapwood for *P. gigantea* inoculated alone and together with *H. annosum*, calculated from scores weighted by size of area occupied. Letters indicate 5% significant differences within rows.

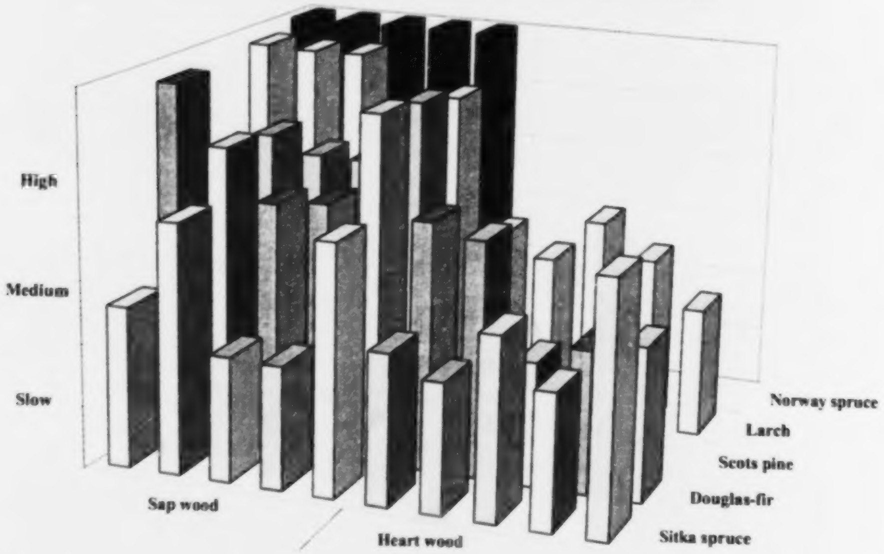
Tree species Growth rate in sap wood	**Scots pine**	**Larch**	**Douglas-fir**	**Norway Spruce**	**Sitka Spruce**
P. gigantea alone	2.6_B	2.9_A	1.7_C	3^*_A	1.5_D
P. gigantea + *H. annosum*	2.3_B	2.8_A	2.4_C	3*	1.6_D

* Due to heavy contamination with moulds, results for Norway spruce are not totally comparable with the other species.

DISCUSSION

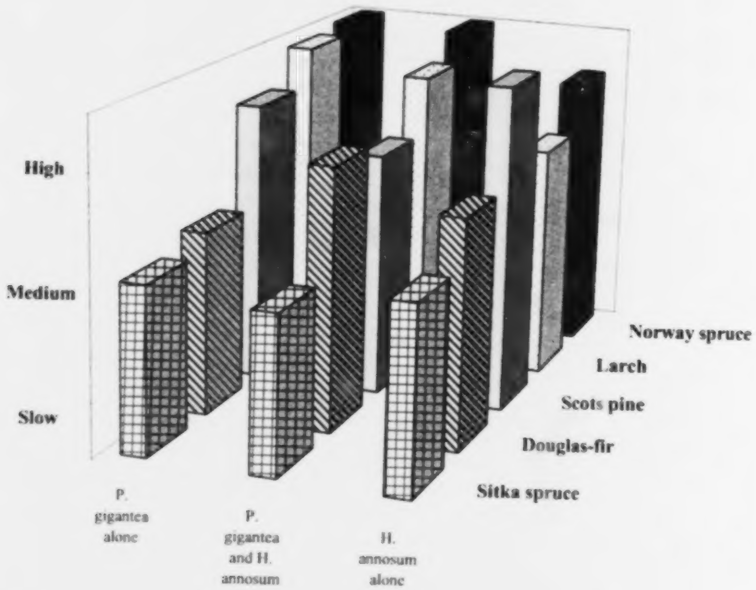
Testing the efficiency of stump treatment under laboratory conditions cannot be compared to *in situ* treatments. However, laboratory tests are, by comparison, faster and easier to carry out and they may provide valuable information. The purpose of the present study was to investigate whether the wood of Sitka spruce, Larch and Douglas-fir could be colonized by the Rotstop isolate of *P. gigantea*, and whether infection by *H. annosum* was prevented by this application. Failure under optimal conditions in the laboratory might indicate problems with practical application in the field.

Estimating the success of colonisation by assessing the abundance of surface mycelial growth only a few days post treatment may be problematic. However, the three types of mycelial abundance were very distinct, and in addition the size of the area occupied by each growth rate category was calculated carefully. The area weighted score was considered to be the best possible method of quantifying the difference in amount of surface mycelium. How accurately the amount of surface mycelium reflects the suitability of the substrate is of course debatable. In any case, the differences between each tree species were barely significant (at p=0.05), and it is not possible to determine the extent to which these were genuine species responses, or artifacts of a novel system of experimentation.



Abundance of P. gigantea mycelium when inoculated together with H.

Figure 1. Rotstop® was able to prevent infection by H. *annosum* for all tree species independently of growth rate of the active agent (*P. gigantea*) as estimated by the amount of mycelium on the discs' surfaces. In Fig. 1, each column represents one disc. Norway spruce and Scots pine did not have visible heart wood. The average growth rate of both fungi on sapwood of each tree is shown in Fig 2.



Comparison of growth rates in sapwood

Figure 2. Both *P. gigantea* and *H. annosum* grew well on Scots pine, larch, and Norway spruce, less fast on Douglas-fir and slowest on Sitka spruce. As only *P. gigantea* is present on Rotstop treated discs, the growth rates of *H. annosum* is just illustrated by the right column.

CONCLUSION

Wood of larch, Douglas-fir and Sitka spruce is suitable as substrates for *P. gigantea* (Rotstop®). Growth of *P. gigantea* seemed to be slower and surface mycelium less dense on Sitka spruce and Douglas-fir, compared to Larch and Scots pine. *P. gigantea* prevented infections of *H. annosum* on Larch, Douglas-fir and Sitka spruce. However, these were preliminary laboratory tests, and they must be verified by field trials before final conclusions on the suitability of Rotstop for stump treatment of tree species other than Norway spruce and pine.

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POTENTIAL FOR BIOLOGICAL CONTROL OF *HETEROBASIDION ANNOSUM* IN THE UK USING ROTSTOP®

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SUMMARY

Currently, *Phlebiopsis gigantea* is used as a stump treatment to control *Heterobasidion annosum* in the UK, but is only applied to pine grown in the south east of England. Stumps of other conifers are treated with urea. A possible alternative is Rotstop®, a form of *P. gigantea* registered for use in Scandinavia, and effective both on Norway spruce (*Picea abies*) and pine (*Pinus* spp.). We have examined the genetic diversity of a range of isolates of *P. gigantea* (including Rotstop), and also compared their ability to colonise the sapwood of Sitka spruce (*Picea sitchensis*), Norway spruce and pine. On the basis of the our results, we consider the potential of Rotstop for stump treatment on spruce and pine in the UK.

Keywords: Rotstop, *Phlebiopsis gigantea*, *Heterobasidion annosum*, butt rot, bio-control

INTRODUCTION

The root and butt rot pathogen *Heterobasidion annosum* is the most economically significant disease of conifer forests in northern temperate regions. It occurs in virtually all managed coniferous forests of the Northern Hemisphere and causes losses in Europe which exceed €790 million *per annum* (Woodward et al. 1998). To control the disease, the stumps of freshly felled trees are protected with prophylactic treatments using either chemicals (Pratt 1996, Pratt et al. 1998, Rishbeth 1959) or with the bio-control agent *Phlebiopsis gigantea*, to prevent invasion by *H. annosum* (Holdenreider and Greig 1998, Rishbeth 1963).

One formulation of *P. gigantea* available for stump treatment is marketed as PG Suspension (Omex Environmental Ltd., Kings Lynn). The isolate it contains came from Scots pine, and PG Suspension is registered for use in the UK, but only on pine. Much earlier work, aimed at establishing whether UK isolates could be effective against *H. annosum* on species other than pine (Rishbeth 1970, 1963), was not pursued. However, in the UK the major commercial conifer species (*c.* 28% forest area in Britain) is Sitka spruce, and only chemical stump treatment is available for this species (Pratt 1996). There is an alternative formulation of *P. gigantea*, Rotstop®, which is registered for use in Scandinavia and effective on both Norway spruce and pine. This could, potentially, provide a non-chemical form of stump treatment for Sitka spruce in the UK.

Therefore, two questions were addressed within this study:

- Can Rotstop be introduced for use in the UK, or is it genetically distinct from the UK population of *P. gigantea*?
- Will Rotstop be effective on Sitka spruce?

The genetic variation within and between Scandinavian and UK isolates of *P. gigantea*, including the current isolates in PG Suspension and Rotstop®, was examined using molecular markers. The colonising ability of some of these isolates was also examined in logs of Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*) and Sitka spruce (*P. sitchensis*).

MATERIALS AND METHODS

Molecular analysis

Cultures of *P. gigantea* (see Table 1) were maintained on 1.5% Oxoid malt agar at 20°C in the dark. DNA was extracted from isolates grown for seven days on MA overlain with cellophane discs. The mycelium was scraped from the cellophane and the DNA extracted by grinding in Tris-HCL, EDTA and SDS using an electric drill (Kim et al. 1999). Molecular markers were generated using RAPD-PCR with Operon primers obtained from VH Bio (Newcastle UK) and also by using RAMS-PCR (randomly amplified microsatellite DNA) as described by Vainio et al. (1996) to characterise the isolates. The banding patterns were visualised on 1.4% agarose gel formulated within Tris-acetate-EDTA (TAE) buffer, incorporating ethidium bromide, and viewed on a GelDoc 1000 system (Bio-Rad, Hemel Hempstead, UK).

Colonising ability

Two weeks before inoculation into logs, autoclaved wheat grains were soaked in sterile distilled water and colonised with one of four UK or four Scandinavian isolates of *P. gigantea*. Fourteen 0.5 m logs each of Scots pine, Norway spruce and Sitka spruce were obtained from a site near Alice Holt Lodge in Surrey (Nat. Grid reference SU 807427) in February 2001. The cut ends of all logs were coated in a bitumen-based sealent (Isoflex, Wetpatch) immediately after felling and then inoculated with the pre-colonised wheat grains the following day. Using a balanced incomplete block design, each log of each species was inoculated with four of the eight isolates. Wheat grains were dropped into holes drilled through the bark with a power drill, at four equally spaced points along a central band, equidistant from the cut ends of each log. The logs were maintained at 18-20°C and after four weeks, the bark was removed from around the inoculation points, and extent of growth assessed by tracing the visibly colonised areas (lesions) on both the sapwood and the inner surface of the bark. The traced lesions were cut out, weighed, and the weight converted to an area measurement.

RESULTS

Molecular analysis

Five of the 22 RAPD primers tested produced clear, consistent banding patterns (OPA02, OPA04, OPA13, OPA18 and OPK19) and a considerable degree of polymorphism was apparent in the RAPD markers. RAMS markers showed similar levels of polymorphism. This indicated a high level of variation within the *P. gigantea* UK population. However, Scandinavian isolates (including Rotstop) had many of the molecular markers seen in the UK isolates, and no distinct grouping emerged between isolates from different geopgraphic areas. Typical molecular profiles and a dendrogram are shown in Fig. 1.

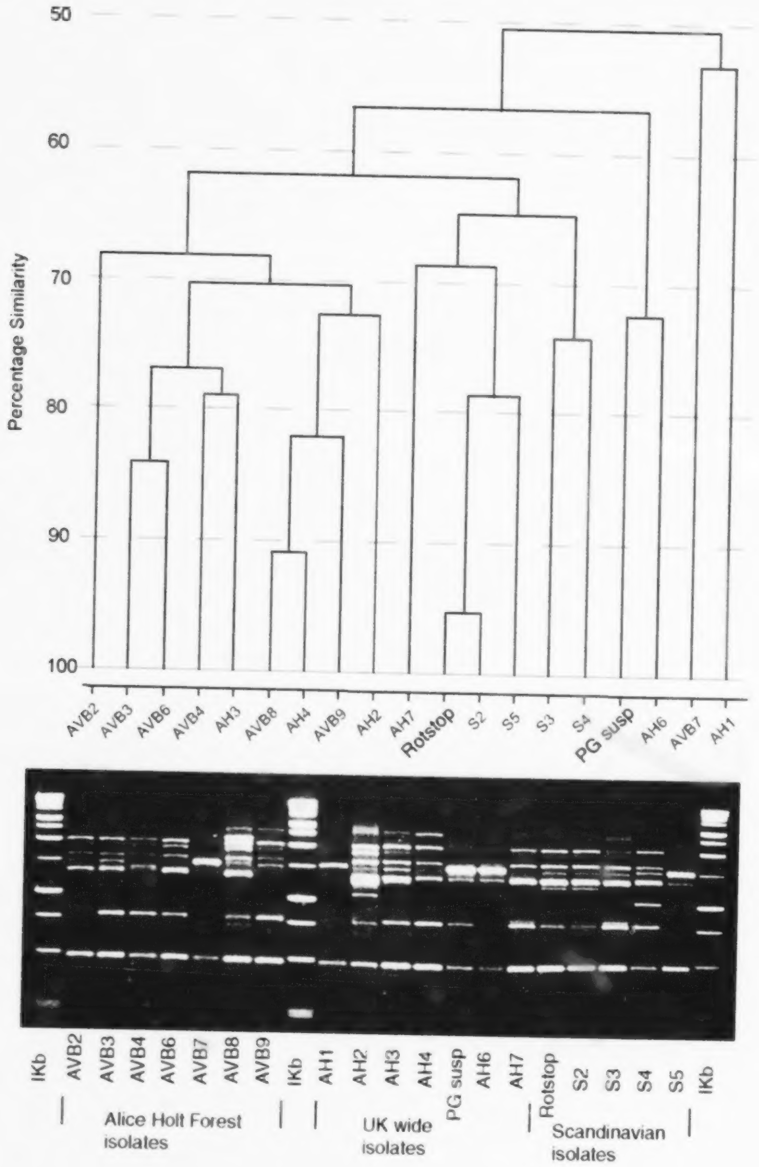

Figure 1. RAPD profiles of UK and Scandinavian isolates of *P. gigantea* following amplification with OPA13, and dendrogram produced from analysis of all molecular markers generated by five Operon primers.

Colonising ability

There was marked size variation in the *P. gigantea* lesions visible on the surface of the sapwood, both between ($F_{(2,18)}=168.25$; $p<0.001$) and within the three host species ($F_{(21,105)}=3.34$; $p<0.001$; see Figs. 2 and 3). Lesions over 4000 mm^2 were recorded in Scots pine logs, whereas in Norway spruce and Sitka spruce lesions were, on average, around 6 times smaller. However, there was no overall difference between the ability of the Scandinavian isolates and the UK isolates to colonise Sitka spruce compared with Norway spruce, and indeed the isolate from PG Suspension was apparently just as effective at colonising the sapwood of Sitka spruce as the Rotstop® isolate.

DISCUSSION

The aims of this study were twofold. Before embarking on efficacy trials with Rotstop® in the UK, there had to be consideration of whether the isolate of *P. gigantea* that it contains was genetically distinct from UK populations of *P. gigantea*. Secondly, it was necessary to assess the ability of Rotstop® to colonise Sitka spruce, the most economically significant UK forestry crop. RAPD analysis demonstrated that all the isolates tended to share at least 50% homology in molecular markers, and although the DNA profiles indicated a relatively high degree of population variation within this, Rotstop® and the other Scandinavian isolates, did not form a separate cluster, but fell within the range of variation normally seen for UK isolates.

As the UK samples were all originally isolated from pine species, whilst the Scandinavian isolates were taken from Norway spruce, it had been thought the latter might be more effective colonisers of Sitka spruce. However, all of the isolates tested, regardless of their geographic origin, were much more effective at colonising pine than either Norway spruce or Sitka spruce. There was no significant difference in the overall performance of the isolates between the two spruces, and no significant variation in performance of the isolates under test within either species.

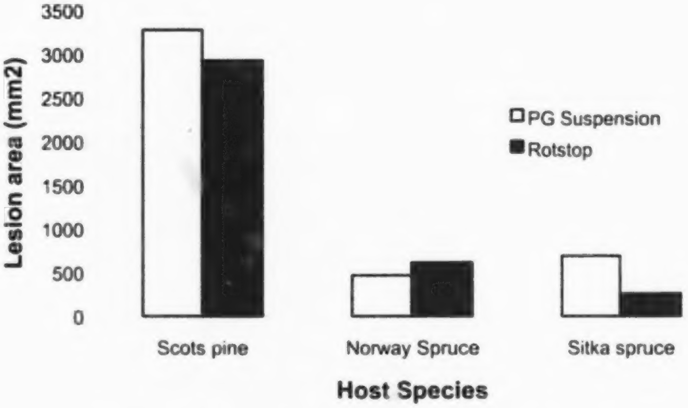

Figure 2. Mean lesion size of UK and Scandinavian isolate of *P. gigantea* on three host species.

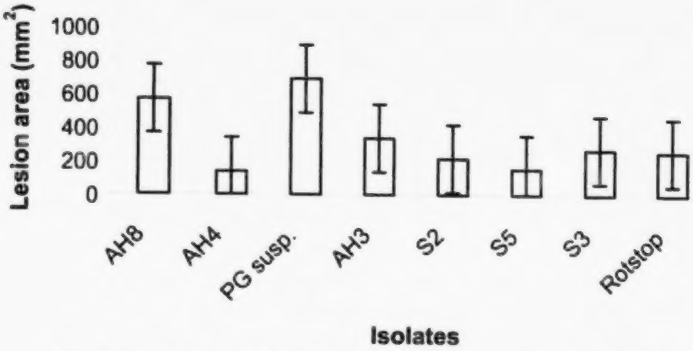

Figure 3. Mean size of *P. gigantea* lesions in Sitka spruce sapwood (error bars indicate +/- se).

CONCLUSION

Isolates of *P. gigantea* from Scandinavia and the UK appear to comprise a single biological species so Rotstop could be used in the UK. In addition, some individuals of *P. gigantea* which originate from pine in the UK appear to be just as effective at colonising spruce as Rotstop, and the other *P. gigantea* individuals isolated from spruce in Scandinavia. However, further work is needed to assess the ability of *P. gigantea* isolates to prevent colonisation of pine and spruce stumps by *H. annosum* in field trials.

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RESULTS OF *HETEROBASIDION ANNOSUM* ERADICATION PERFORMED IN 1993-94 IN TWO RED PINE PLANTATIONS

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An eradication trial took place in two 50- and 60-year-old red pine plantations. The larger one is covering 80 ha and eight infection centres have been treated in 1993. The 2 ha plantation had only one centre treated in 1994. All red pines inside each infection centres, plus one row of apparently healthy red pines around these centres were marked. All marked trees were harvested and stumps were removed, carried to a dump or buried on site and covered with one metre of sand. Also, the top soil and the organic matter were scraped away from the surface and buried on the site under 1 m of sand. All these clean area were planted with 2-year-old red pine seedlings as bioindicators. These seedlings and trees surrounding the centres have been inspected every year since until year 2000. *H. annosum* has been detected on two seedlings in each of the three areas treated, but was not present on the surface of the six others. Diseased trees on the surrounding boundaries of the centres were detected on five sites out of nine; red pine, white pine, balsam fir and black spruce were found infected. Finally, a new infection centre were localized in 1998 following the death of a red pine. It is suggested to maintain a quality control during the eradication work to ensure that all organic material is buried. It is also recommended to remove a second row of apparently healthy red pines around the centre when this is not causing a high probability of windthrow.

BIOLOGICAL CONTROL OF *ARMILLARIA* SPP. WITH *BASIDIOMYCETES*

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Armillaria root rot is one of the most significant diseases in Polish forests. In this study, 30 isolates of different *Basidiomycetes* were tested as a biological control of *Armillaria* spp.: *Bjerkandera adusta, Hypholoma fasciculare, H. sublateritium, Kuehneromyces mutabilis, Lentinus lepideus, Pholiota squarrosa, Pleurotus ostreatus, Pluteus atricapillus* and *Trametes versicolor*. In laboratory experiments, isolates of different species or inside each species showed significant differences in growth rate, wood decay ability, influence on the growth of *Armillaria* spp. *in vitro*, and speed of wood colonisation. The best isolates of each species were used to treat deciduous tree stumps. Some species could exclude *Armillaria* spp. from stumps. *Pholiota squarrosa* and *Lentinus lepideus* colonised stumps very slowly. The best results have been obtained on treated stumps after commercial thinning. *Hypholoma fasciculare, H. sublateritium, K. mutabilis* and *P. ostreatus* colonised stumps very fast. One year after inoculation, fungi were present in whole stumps.

VARIATION IN *PHLEBIOPSIS GIGANTEA* AND MONITORING THE EFFECTS OF RELEASE

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Phlebiopsis gigantea (Fr.) Jülich is a common saprophytic and decay basidiomycete fungus in coniferous forests and has been used as a competitor biological control agent against the pathogenic fungus *Heterobasidium annosum* (Fr.) Bref., which causes serious root and butt rot diseases in conifers. The treatment of the cut surface of stumps with *P. gigantea* prevents the penetration of pathogen into the stump and its spread to the uninfected trees. So far, treatment has usually involved a single genotype of the biocontrol agent which has widely applied across large geographic area. It is important to evaluate the ecological risk of such a large-scale application on the native populations of this fungus and other stump colonizers. The initial requirement of such a study is to be able to detect and monitor the released isolate.

Here we used mitochondrial DNA polymorphisms for strain discrimination of *P. gigantea*. Single basidiospore isolates obtained from 50 fruiting bodies collected from two sites with 30 km distance, in non-treated pine forests around Uppsala, Sweden, showed highly variable mitochondrial–RFLPs, almost specific at individual level and all were distinct from that obtained from the commercial isolate, RotStop. In order to investigate the source of this high polymorphism, inheritance of mitochondrial DNA was studied, *in vitro*, by mating homokaryons possessing different mitochondrial genotypes in petri plates. Heterokaryosis was detected with the use of Amplified Fragment Length Polymorphism (AFLP) on nuclear DNA. The model of transmission of the mitochodrial genome was then elucidated in the newly formed heterokaryotic mycelia, fruiting bodies and progenies.

INTIMATE MIXTURES OF SUSCEPTIBLE SPECIES AND THE SPREAD OF ARMILLARIA ROOT DISEASE

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Total stem maps in nine young stands (0.25 to 2.1 ha in size) with *Armillaria ostoyae* (Romagn.) Herink root disease and consisting of intimately mixed susceptible conifer species typical of older plantations in the southern interior of British Columbia were prepared. It was shown that for many tree species, the identity of an infected tree affects the incidence of infection of the various species in its immediate neighbourhood. In mixtures with a significant component of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and western larch (*Larix occidentalis* Nutt.), of western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and Douglas-fir, and of lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Englem.) and western hemlock, spread between adjacent trees of the same species was more common than spread between adjacent trees of different species. However the phenomenon was only evident in seven plantations over 14 years of age, and not in two plantations less than 10 years old, even though the latter also had intimate mixtures of these conifer species. It may be that differences in rooting habits of these conifer species result in more frequent root contact between trees of the same species, and hence more rapid spread of the pathogen. The results suggest that intimately mixed plantations of susceptible conifers may sustain less damage from Armillaria root disease than the same species in pure plantations.

Genetics and Population Dynamics

MOLECULAR MARKERS REVEAL GENETIC ISOLATION AND PHYLOGEOGRAPHY IN THE S- AND F-INTERSTERILITY GROUPS OF *HETEROBASIDION ANNOSUM*

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SUMMARY

Phylogenetic relationships were studied in the S- and F-groups of *Heterobasidion annosum* Fr. Bref. using P-group isolates as outgroup. In all, 29 fungal isolates were included from Europe, Asia and western North America. The DNA sequence was obtained for five different loci; calmodulin, glyceraldehyde 3-phosphate dehydrogenase, heat stress protein 80-1, elongation factor 1-α, and an unidentified target sequence Ha510. The results from the five loci were largely univocal and the combined data set gave strong support for the separation of the European F-group, the Euroasian S-group and the North American S/F-group being phylogenetically separated. The conclusion was that the F group had separated from the other two first, and that European S and North American S/F are sister clades. No indication of recent hybridisation between European S and F was obtained.

INTRODUCTION

Heterobasidion annusum is, in economical terms, the most important disease of conifers of temperate regions. It occurs in managed coniferous forests of the northern hemisphere, as far north as central Finland and south as far as northern Africa and Central America.

H. annosum was long considered to be a single species with a wide ecological range. However, mating experiments carried out since the 1970s have revealed the occurrence of intersterile (IS) groups within *H. annosum* (Korhonen 1978; Chase and Ullrich 1988; Capretti et al. 1990; Stenlid and Karlsson 1991). Three groups (S, F and P) are found in Europe and two (S and P) in North America. The IS groups show difference in distribution and host preference although the host ranges can be wide and overlapping. The P group occurs mostly in pine forests but is able to attach many other tree species (Korhonen 1978). The S group attacks many tree genera in North America, but occurs almost only on *Picea abies* in most parts of Europe; however, it is found to attack *Abies sibirica* in north-eastern Europe (Korhonen et al. 1997). The F group is a pathogen or saprophyte on *Abies* spp. and is confined to central southern Europe (Korhonen et al. 1998). Differences in RAPD and isozyme patterns indicate genetic isolation of the IS groups (Karlsson and Stenlid 1991; Otrosina et al. 1993; LaPorta et al. 1997). Physiological as well as biochemical characters, in addition to different morphology and intersterility, have led to the separation of the *H. annosum* complex into three different species; *H. abietinum*, *H. parviporum* and *H. annosum*, representing the S-, F- and P-groups, respectively (Niemelä and Korhonen 1998).

Although hybrids are rarely found in nature, the IS groups of the *H. annosum* complex are not completely intersterile. In mating tests carried out in the laboratory, homokaryotic pure cultures from different groups mate with each other in variable frequencies. In Europe, the compatibility of isolates from the P group with S and F isolates respectively, is low (Stenlid and Karlsson 1991). However, the compatibility between groups S and F is 25-75%, depending on the origin of the S isolates (Korhonen et al. 1992; 1997). In a study of interfertlilty of sympatric populations of S and F type from central Europe, about 24% of the pairings were interfertile based on dikaryon formation, while parings between northern European S strains and southern European F strains were about 72% interfertile (Korhonen et al. 1992). This indicates that the mating tests are not enough to separate the F and S IS-groups into biological species.

Knowledge of population structure and evolution of the *H. annosum* complex helps our understanding of host specialization. Two alternative explanations for the splitting of the S and F groups are previously proposed (Korhonen et al. 1997). The first hypothesis implies a relatively recent differentiation of the S and F IS groups. During the ice age, *P. abies* found refuges in Southern Russia, in the Balkans and, to a small degree, in southern Italy (Schmidt-Vogt 1977), while *Abies alba* survived in some enclaves in the Balkan peninsula as well as in southern Italy (Kral 1979). After the glacial period, the colonization of northern and central Europe by *P. abies* took place from Russia, the Carpathian mountains and the Balkan peninsula, whereas the migration of *A. alba* into central Europe involved mainly the Italian refuge (Bernetti 1995). It is possible that the differentiation of the F group took place in the southern Italian refuge of *A. alba*. An alternative hypothesis is that the differentiation of the IS groups relates to the migration of their main host species. *Abies* and *Picea* have migrated to Europe from eastern Asia. One population of *H. annosum* may have followed *A. sibirica* or *P. abies* along the northern route through Siberia. It adapted to attack both these trees and became the S type. The F type possibly developed on the southern migration route of *Abies* where *Picea* may have been absent or rare. Later, in Europe, *P. abies* and *A. alba* came in contact with each other but their specialized root pathogens, the S and F groups of *H. annosum*, were unable to attack effectively the new potential host trees. The hybrids between S and F perhaps would show a reduced fitness which favoured the development of a genetic breeding barrier between the sympatric S and F populations in central and south-eastern Europe.

Several attempts to resolve the species complex by using ribosomal DNA have been made (Kasuga et al. 1993; Garbelotto et al. 1996; Harrington et al. 1998). In the study by Harrington et al., phylogenetic analyses of the *H. annosum* complex based on sequences of the internal transcribed spacer (ITS) and the intergenic spacer region (IGS) of the nuclear rDNA, delimited three major lineages within the complex: the American P form, the European P form and the "fir form", including isolates of both the American S as well as European S and F IS groups. Within the fir clade, minor variation was found in both the ITS and IGS sequence, and both DNA regions revealed three rather weakly supported subclades: i) the S group isolates from North America, ii) Asia and iii) the S and F group isolates from Europe. Little variation was found in the sequences of the fir clade isolates from Europe, and S and F type isolates could not be distinguished, which they interpret as though the two IS groups have just recently diverged and thus do not yet show fixed mutations in the IGS and ITS sequences. In contrast, reconstructed phylogeny based on partial amino-acid sequences of putative manganese peroxidase (MnP) genes from *H. annosum* and its closest relatives indicate that the F group diverged at an early state from the rest of the S group (Maijala 2000). However, these results were not strongly supported, and furthermore, since peroxidases are involved in the biodegradation of lignin in white-rot fungi, the differences in the MnP genes might have resulted from the selective effect of growth in different hosts.

Compatibility of different gene genealogies can be used to infer presence or absence of mixis in a population, and delineate reproductively isolated groups within sexual taxa. Full compatibility among genealogies indicate complete asexuality and incompatibility indicating mixis. This provides the basis for the *Genealogical Concordance Phylogenetic Species Concept* (GCPSC). GCPSC was originally used to investigate recombination in *Escherichia coli* (Dykhuizen and Green 1991), and has in recent years been used successfully to define phylogenetic species limits in several fungal genera (reviewed by Taylor et al. 2000). In this study we developed a multiple gene genealogical approach to understand the evolutionary history of the S and F groups of *H. annosum*. First, we wanted to verify reproductive isolation of *H. annosum* S- and F-groups. Second, we wanted to imply the phylogeographic pattern of differentiation of the IS-groups.

MATERIALS AND METHODS

Materials and culture methods

Fungal isolates, their geographic origin, host species and IS-affinity are shown in Table 1. Mycelia for DNA extraction were grown for 1 week on liquid Hagem media (Stenlid 1985), at 21°C in darkness.

DNA manipulations

DNA was extracted from lyophilized mycelia of each isolate using a standard CTAB method (Johannesson and Stenlid 1999). Fragments of four nuclear genes (calmodulin, glyceraldehyde 3-phosphate dehydrogenase, heat stress protein 80-1, and elongation factor 1-α) and one unidentified target sequence (Ha510), were used as molecular markers. Oligonucleotide primers designed to amplify segments of the three first mentioned genes are presented in Johannesson et al. (2000). To obtain an increased specificity, new primers were created for the elongation factor 1-α locus (forward 5′ TCAACGTGGTCGGTGAGCAGGTA-3′; reverse 5′-AAGTCACGATGTCCAGGAGCATC-3′) by sequencing a subset of *H. annosum* strains with the primers presented in Johannesson et al. (2000). The unknown sequence marker were developed by the method of sequencing with arbitrary primer pairs (SWAPP, Burt et al. 1993) and presented by Ihrmark et al. (2001). Each PCR reaction contained approximately 1 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5-1.7 mM MgCl2, 0.2 μM of each primer and 2 mM of each dNTP in a total volume of 50 μl. PCR amplifications were performed using a Perkin-Elmer Cetus DNA thermal cycler (GeneAmp 2400), under the following conditions: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 52-64°C and 30 s at 72°C, with a final extension of 7 min at 72°C. PCR products were purified using the Qiaquick PCR purification kit (QIAGEN Inc.) prior to sequencing. All sequences were determined with an Applied Biosystems 310 sequencer using the Taq DyeDeoxi Terminator^{TM} cycle system (Perkin-Elmer). Sequences analysis and alignment were performed manually with the help of the program SEQED from the Genetics Computer Group (GCG) Sequence Alignment Software Package Version 7.1 (Devereux et al. 1984). Only unambiguous alignments were used in the phylogenetic distance analyses. Sequence alignments are available from the first author upon request.

Data analysis

Phylogenetic analyses of individual markers and all five markers combined were performed with PAUP 4.0 (Swofford 1999). Maximum parsimony trees were identified using heuristic searches using the tree bisection-reconnection (TBR) branch swapping algorithm. All characters were of equally weight, unordered, and gaps were included in the study as one character/gap. Statistical support for phylogenetic grouping for individual genes and the combined data set was assessed by bootstrap analysis using 1000 replicate data sets with the random addition of sequences during each heuristic search. Earlier phylogenetic studies of the genus (Harrington et al. 1998; Maijala 2000), suggests that the European and American P-groups are basal to the S- and F- group, and the two isolates belonging to the P-IS group (Table 1) were selected as outgroups.

Table 1. Isolates of *Heterobasidion annosum* used in the study.

Isolate	Geographic origin	Host	Collector[a]	IS-G
Br518c2	Brynge, Sweden	*Picea abies*	JS	S
87-179c2	Norway	*Picea abies*	HS	S
FSE-3 (821201-3)	Helsinki, Finland	*Picea abies*	KK	S
95151	Ural, Russia	*Picea abies*	KK	S
95123	Ural, Russia	*Picea abies*	KK	S
95156	Ural, Russia	*Picea abies*	KK	S
OH2-2c3	Switzerland	*Picea abies*	OH	S
Fas1	Finland	*Picea abies*	KK	S
Fas16 (880514.1.1)	Münich, Germany	*Picea abies*	HM	S
Fas13 (871104.5.1)	Vicenza, Italy	*Picea abies*	KK	S
Fas11 (871104.3.1)	Vicenza, Italy	*Picea abies*	KK	S
OH2-8c6	Switzerland	*Picea abies*	OH	F
Faf 3-5 (871007.3.2)	Toscana, Italy	*Abies alba*	KK	F
Faf 5.2 (871005.1.3)	Toscana, Italy	*Abies alba*	KK	F
Faf 7.1 (871007.2.1)	Toscana, Italy	*Abies alba*	KK	F
Faf 4.6 (870900.1.2)	Toscana, Italy	*Abies alba*	KK	F
Faf 6.2 (871005.6.2)	Toscana, Italy	*Picea abies*	KK	F

Faf10.2 (880805.1.1)	California, USA	*Abies sp.*	KK	S
Bc3-3.2	Victoria, BC, Canada	*Tsuga heterophylla*	JS	S
Bc1-4	Victoria, BC, Canada	*Tsuga heterophylla*	JS	S
ORE103	Oregon, USA	*Abies concolor*	TC	S
Tc122-11	Alaska, USA	*Airborne*	TC	S
B1142	Ajusco, Mexico	*Abies religiosa*	DR	S
B1295	Changbai, China	*Abies sp.*	YD	S
B1314	Changbai, China	*Populus sp.*	YD	S
B1092	Katashina, Japan	*Abies mariesii*	KA	S
B1081	Kamikawa, Japan	*Abies sachalinensis*	TY	S
32-1	Vermont, USA	*Pinus resinosa*	TC	P
16-4	Sätuna, Sweden	*Picea abies*	JS	P

[a]JS, J. Stenlid; KK, K. Korhonen; H. Marxmüller; OH, O. Holdenrieder; TY, T.Yamaguchi, KA, K. Aishoma, DR; D. Rizzo, YD; Y. Dai ; HS, H. Solheim

RESULTS

All loci were successfully amplified and sequenced from all isolates of Table 1, with a few exceptions: the calmodulin-locus was not possible to amplify from the American P isolate (32.1), probably because a lacking target region for annealing of the primer camF. Furthermore, the unknown target region Ha510, originally developed for S group strains of *H. annosum*, was not successfully amplified from any of the P-isolates 32.1 and 16.4.

Of the 2062 unambiguously aligned sites, a total of 200 were polymorphic and 130 were informative (Table 2).

Table 2. Statistics of the cladistic analysis of the five loci and the combined data set.

Information	Locus					
	510	hsp	gpd	efa	cam	all
Total no of alignable characters	411	440	471	411	329	2062
No of variable characters	21	47	58	46	28	200
No of informative characters	14	39	30	26	21	130
No of trees	5	1	2	1	4	25
Tree length	23	52	70	52	35	244
Consistency Index (CI) for first tree	0.913	0.9615	0.9286	0.9615	0.8571	0.8852
Retention index (RI)	0.9759	0.9891	0.9728	0.9858	0.9662	0.9621

All genealogies based on the nuclear genes revealed three relatively well supported branches separating i) European and Asian S group isolates, ii) S-group isolates from America and iii) F group isolates. However, incongruences in evolutionary relationship of the three groups were found between the genealogies, although none of the genealogies would support a relatively recent split of the F- and S-groups. The phylogeny reconstructed from the unknown locus (Ha510) was not well resolved.

The combined gene genealogical analysis demonstrated overall similarity to the phylogenetic relationship derived from individual genealogies (Fig 1). As expected, the three groups found in the individual trees were found also in the combined tree. Furthermore, the branch separating of the F-group isolates from the American and European/Asian S-groups was quite well supported, with a bootstrap value of 84.

DISCUSSION

Our data clearly support that the S- and F-IS groups are reproductively isolated. They also support the hypothesis of an early separation between the two IS groups, indicating that the two IS groups have followed their host tree species for a considerable time period.

Harrington et al. (1998) interpreted their data as if the S-group of North America is older than the "fir clade" from Europe and Asia, including both the S and F groups.

The identical rDNA spacer regions in European S- and F-strains may have resulted from a hybridization event in Europe, and along with the concerted evolution and homogenization of the nuclear ribosomal genes, only one of the parental ITS or IGS types has emerged. However, the ribosomal spacer regions constitute only one locus and the accumulated evidence now available support a different inference of the evolutionary history in the *H. annosum* complex.

One inference one can make from our combined data set is the following: the basal split in the F-S complex seems to be European F group differentiating from the rest of the S-F clades (bootstrap support 84). This preceded the well supported separation of the Euroasian S from the American S/F, which form sister clades in the analysis. Subsequently, there seems to have been some differentiation on the two continents; Japanese S/F differ from the rest of Euroasian S, and Mexican S/F differ from the US and Canadian isolates. The overall pattern is consistent with variation in Manganese peroxidase (Maijala, 2000) and Laccase (Abu et al , in prep.) Furthermore, the morphological differences between the European S- and F-groups as well as differences in pecitinase isozymes, indicate a long lasting separation and is not compatible with the hypothesis of recent hybridisation.

Our findings support the idea that the Euroasian S, The European F and the North American S/F are three distinct phylogenetic species. What we interpret as different phylogenetic species are clearly separated into distinct clades using the four protein markers and they share the same neutral alleles at the locus 510. Genetic isolation precedes the loss of shared polymorphism, and the proportion of loci for which the polymorphisms are shared changes in inverse proportion to the time since genetic isolation began. The loci sort randomly due to genetic drift. Therefore, in genetically isolated species one would expect to find some variable nucleotide positions where polymorphism is still shared by sibling species, other positions where only one species is polymorphic and the other is fixed for one allele, and others where both species have fixed loci. However, it seems to be rare that both species still share polymorphism and the concern can be reversed to make the argument that discovering a few loci, or even one, that shows fixation in one or the other of the phylogenetic species is evident for genetic isolation (Taylor et al. 2000).

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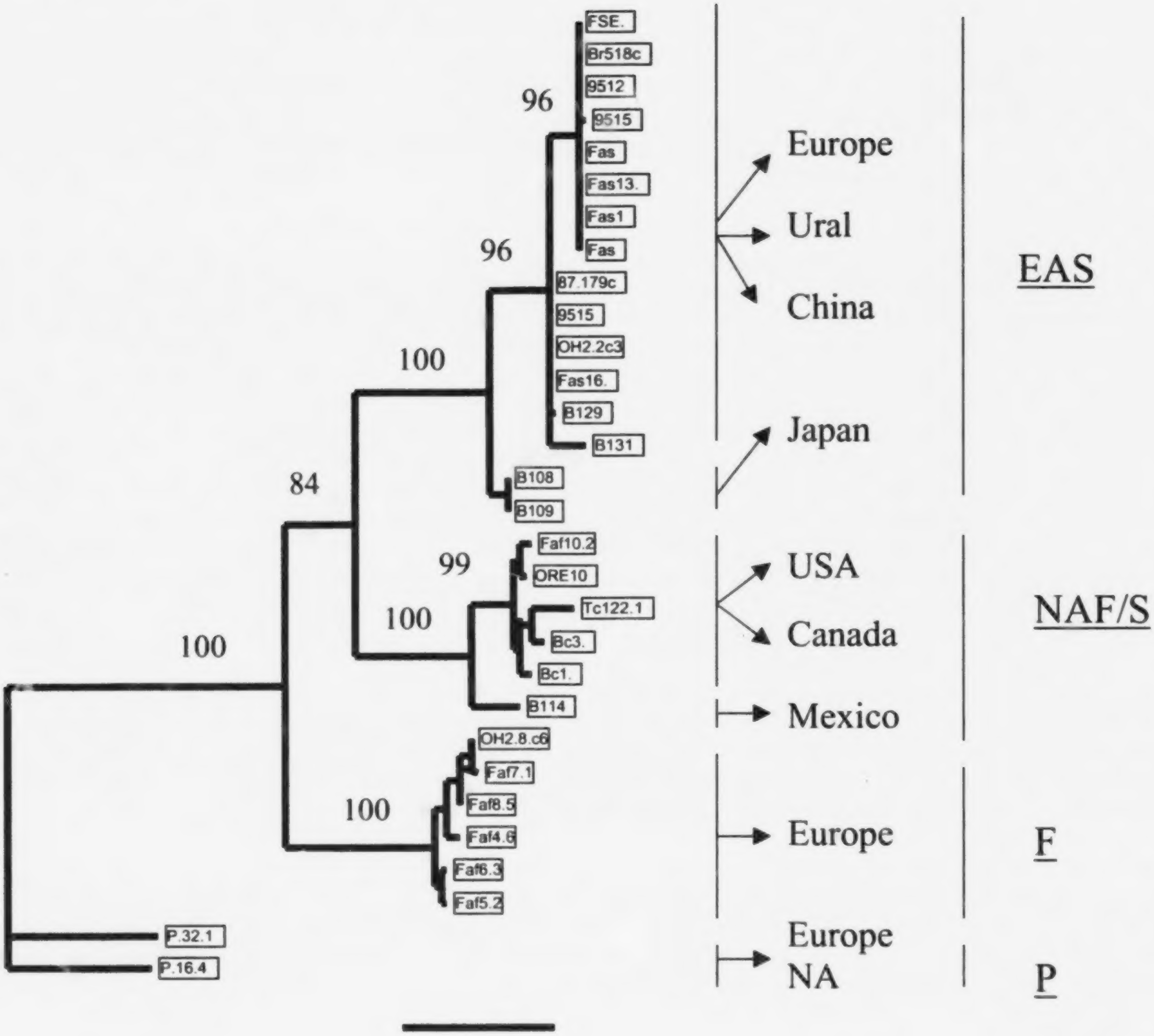
96
96
100
84
99
100
100
100
FSE.
Br518c
9512
9515
Fas
Fas13.
Fas1
Fas
87.179c
9515
OH2.2c3
Fas16.
B129
B131
B108
B109
Faf10.2
ORE10
Tc122.1
Bc3.
Bc1.
B114
OH2.8.c6
Faf7.1
Faf8.5
Faf4.6
Faf6.3
Faf5.2
P.32.1
P.16.4
Europe
Ural
China
Japan
USA
Canada
Mexico
Europe
Europe
NA
EAS
NAF/S
F
P

Figure 1. One out of 25 most parsimonious trees for S- and F-group isolates of *H. annosum* using two P isolates as outgroup. The analysis was based on a combined data set of 130 informative characters from the DNA sequence of the following loci: calmodulin, glyceraldehyde 3-phosphate dehydrogenase, heat stress protein 80-1, elongation factor 1-α, and an unidentified target sequence Ha510. The length of the branches correspond to the number of nucleotide differences, reference bar = 10 substitutions. Bootstrap values above 80 are indicated.

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STUDIES ON THE ECOLOGY AND GENETICS OF HYBRIDIZATION IN *HETEROBASDION*

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The *Heterobasidion* taxonomic complex comprises two of the most important fungal tree pathogens in North America (Otrosina 1989), and includes at least three other taxa in Eurasia (Niemela 1998). These taxa were initially identified as intersterility groups (ISGs) (Korhonen 1978) within the species *H. annosum*, but the current trend is to assign them the species rank based on molecular, morphological, and host-association traits. European ISGs have already been accepted as different species (Niemela 1998). Although yet to be defined as species by taxonomists, the North American *Heterobasidion* ISGs have a much wider genetic divergence and isolation than their European counterparts (Otrosina 1993), and are also ecologically specialized on different hosts (Otrosina 1989; Worrall 1983). In this paper we will use the terms "species" and "ISGs" interchangeably when referring to the two North American taxa of Heterobasidion.

In California, host specificity effectively strengthens the genetic isolation between the two sympatric *Heterobasidion* ISGs. Isolates of the "S" ISG infect mostly true firs, hemlocks, and sequoias; the "P" ISG infects mostly pines, junipers, and incense cedars (Otrosina 1989). Nevertheless, there is significant interfertility among S and P isolates in laboratory mating tests (Harrington 1989), where host-specific discrimination is artificially removed.

We have recently gathered evidence for hybridization between the North American S and P ISGs. In an analysis of 7 loci of 51 S and 34 P isolates from California (data not shown), allelic frequencies ranging between 0.01-0.11 in one ISG and 0.86-1 in the other were suggestive of inter-ISG introgression in areas where both ISGs coexist (Garbelotto et al., in prep.). Outside the areas of sympatry, frequencies of putatively introgressed alleles are close to zero. Moreover, a first generation hybrid genotype was found in a mixed ponderosa pine-Western juniper stand in Northeastern California (Garbelotto 1996). The hybrid had colonized at least three neighboring trees and one stump, and time since initial colonization was estimated to be 5-25 years. This is the first stable natural *Heterobasidion* hybrid reported in the literature.

The potential ecological and evolutionary consequences of hybridization are widely recognized. Recent studies have provided evidence of past hybridization events in fungi (Tsai 1994; O'Donnell 1997; Brasier 1998, 1999), and have indicated that this process may be a viable mode of fungal speciation. Hybridization can be repressed either by the incompatibility of different mating systems (prezygotic isolation) or, at the cellular level, by low hybrid vigor due to the presence of two incompatible non co-adapted genomes in the same individual (unconditional postzygotic isolation) (Brasier 1995; Orr 1995).

These hypotheses do not entirely explain the repression of hybridization in the *Heterobasidion* complex, considering the significant compatibility between the North American taxa in the laboratory (Harrington 1989) and the stable nature of artificial and natural hybrids (Harrington 1989; Garbelotto 1996). A significant correlation has been shown between pathogenicity and type of mitochondrial genome, by means of inoculation of laboratory hybrids on pine germlings (Stenlid 2001), nevertheless, no information is available on the overall fitness of hybrids.

In this paper, we present experiments designed to test the following hypotheses:

A) Hybrids are genetically stable, in spite of the known potential for genomic instability within the species belonging to the complex

B) Hybrid host range is not larger than that of parental types, nonetheless hybrids are as competitive as parental types and are capable of surviving in nature

C) Presence of stumps and changes in forest composition increase sympatry of the North American *Heterobasidion* species.

MATERIALS AND METHODS

Studies on the genetic stability of hybrids

It has been shown that in *Heterobasidion* heterokaryons, each parental nuclear genome can be found by itself in a homokaryotic hyphae within the thallus (Hansen 1994). Individual parental genomes can also be recovered by subculturing unicellular conidia. It has also been shown that the more genetically unrelated the parental nuclei are, the greater the tendency of a heterokaryon genotype to produce uninucleate conidia. This observation has been interpreted in terms of "genomic conflict" between two genetically different parental nuclei (Ramsdale 1994).

The presence of genomes belonging to two different species in a hybrid genotype, should result in maximum levels of instability due to genomic conflict. In order to investigate the genetic stability of hybrids, we studied the nuclear composition of a) hyphae in the thallus and b) of conidia produced by the natural hybrid genotype retrieved in California. Subcultures were grown on cellophane overlaid on standard malt extract agar (MEA) and a total of 100 individual hyphal tips were subcultured. All subcultures were analyzed for presence of clamps and typed as S, P or hybrid SP, by Taxon-Specific Competitive-Priming (TSCP) PCR (Garbelotto 1996).

Conidia of three isolates (Table 1) were stained with DAPI and analyzed at 300X magnification under fluorescent lighting. For each isolate, the number of uni-, bi- and multi-nucleate conidia was tabulated.

Table 1. Recovery of uninucleate, binucleate, and multinucleate conidia from three isolates of *Heterobasidion annosum*.

				Uninucleate conidia		**Binucleate conidia**		**Multinucleate conidia**[c]	
Fungal isolate	**Nuclear status**	**ISG**[a]	**N**[b]	**No.**	**%**	**No.**	**%**	**No.**	**%**
L2.8.R1.a	Homokaryon	S	188	61	32	104	56	23	12
L2.7.R5	Heterokaryon	S	187	108	58	74	39	5	3
AWR400	Heterokaryon	S-P	194	135	70	55	28	4	2
Chi-square	*P<0.001*[d]	*d.f=2*							

[a] ISG = Intersterility group.

[b] N = total number of conidia sampled.

[c] Multinucleate = 3 or more nuclei per cell.

[d] Distribution of nuclei are significantly different among the three isolates. Pairwise comparisons of uninucleate conidia distribution (Z-tests):
L2.8.R1.a-L2.7R5, Z = 4.92
L2.8R1.a-AWR400, Z = 7.26
L27R5-AWR400, Z = 2.4
Z values for all comparisons are >1.645, and therefore significant at P=0.05.

Finally, thirty individual conidiophores were isolated, and 10 individual conidia from each conidiophore were subcultured. The resulting 300 single-conidium isolates were analyzed for clamps and their ISG was determined by TSCP PCR.

Studies on the pathogenicity and virulence of hybrids: inoculation experiments

We performed two greenhouse experiments and one field inoculation experiment. In greenhouse trials each ISG has shown higher virulence on its corresponding natural hosts (Worrall 1983) (Otrosina et al., in prep.). True firs, sequoias, hemlocks and Douglas-firs ("S-hosts") were more susceptible to S isolates, while pines ("P-hosts") were more susceptible to P isolates. Sitka spruce seedlings were always susceptible to both ISGs, and thus this tree species was identified as a "universal" host.

In greenhouse experiment 1 (Fig. 1), we compared virulence of an S, a P, and the natural SP-hybrid isolate. Although these isolates were genetically unrelated, they each infected several trees and stumps, demonstrating their viability in the field. All three isolates were dikaryotic. Isolates were inoculated on seedlings of white fir (S-host), ponderosa pine (P-host) and Sitka spruce (S and P "universal" host) and virulence cwas mesaured in terms of seedling mortality. In greenhouse experiment 2, ponderosa pine seedlings were inoculated with one S and one P homokaryon and with an SP dikaryon obtained by mating the S and P homokaryons in the laboratory, allowing a direct comparison of virulence. Differences in ploidy may be irrelevant in this species, as both haploids and dikaryons of *H. annosum* can be virulent and are commonly found in nature (Garbelotto 1997).

Finally, we performed a field inoculation experiment (experiment 3, Fig. 1), in which fungus-colonized wood dowels were inserted into holes drilled in white fir (S-host) tree roots. In this experiment, virulence was expressed as the extent of longitudinal colonization of inoculated roots. We used the same S, P, and SP hybrid isolates as in experiment 1. However, in this case holes were drilled beyond the cambium and the outer layer of the xylem; this bypassed the pathogen-specific host defense responses. It has been shown that sapwood inoculations do not normally discriminate between *Heterobasidion* species (Swedjemark 1999). To further verify this assumption, host reaction was also studied through high pressure layer chromatography (HPLC) analysis of colonized root xylem extracted twice with methanol (Bonello 1993).

Stumps as a potential hybridization zone

The question arises whether conditions exist in nature that may be conducive to hybridization. Two main requirements exist for successful hybridization: (a) both species must be present in an area, and (b) there must be colonization courts where both species can come into close contact, mate, and their hybrid offspring thrive. Fresh stumps may provide a non-selective colonization court in which the S and P taxa can mate (Otrosina 1992; Garbelotto 1996).

To quantify the impact of stump availability on the composition of *Heterobasidion* populations, we studied the genetic structure of this fungus in stumps, trees and the air-spora in three California National Forests (NFs) dominated by the P-selective hosts, pine and juniper. Airborne spores provide a measure of the overall population structure in a site. Spores are also essential for the persistence of *Heterobasidion* in nature (Otrosina 1989). Spores were collected by using the exposed wood-disk method described by James and Cobb (1982). Individual colonies were isolated from the wood-disks and their ISG was determined by TSCP-PCR. Isolates from wood and stumps were obtained by isolations both from infected wood and from the context of basidiocarps. ISG was determined by isozyme analysis or by TSCP PCR.

Pathogen species are tracking their specific host(s)

While a common habitat is required for physical contact and mating between the two *Heterobasidion* species, it is also necessary that both species be present in the same geographic location. We analyzed the presence of S isolates in live pine/juniper trees and stumps in relationship to the geographic distance from the two most important hosts for this group: hemlock and true fir (*Tsuga* and *Abies* spp.). In this analysis, we included data from three National Forests in which the disease is known to affect both S- and P-hosts (Inyo, Plumas, Modoc), as well as data from NFs where the disease is known only on S-hosts (Stanislaus, Eldorado) or on P-hosts (Cleveland, data not shown). Regression analyses were performed to verify the presence of a correlation between distance from the specific host and frequency of retrieval of its adapted pathogen.

RESULTS AND DISCUSSION

On the genetic stability of the natural hybrid

All of the hyphal tip subcultures were clamped and heterozygous for the P- and S- specific markers (i.e. they were putative hybrids). This result is explainable by hypothesizing a complete lack of homokaryotic hyphae in the thallus. This feature would differentiate the hybrid isolate from S and P heterokaryons, in which homokaryotic hyphae are always present. This characteristic would also imply a significant stability of the hybrid thallus. Both laboratory experiments (Hansen 1994) and field surveys (Garbelotto 1998) have indicated that natural isolates may be mosaics of the two homokaryotic parents and of the resulting heterokaryon. The stability of the hybrid thallus was also confirmed by multiple isolations (over 20) of the same hybrid genotype from at least four different stems (stumps and trees).

The analysis of the nuclear condition of the conidia provides us with a further understanding of the ploidy modifications associated with the natural hybrid. The number of uninucleate conidia, as determined by microscopic observation after DAPI-staining, was significantly higher in the hybrid than in two S isolates (Table 1, chi, P). Despite a majority of uninucleate conidia in the hybrid, all isolates obtained by subculturing of individual mitospores were S-P hybrids as per TSCP PCR determination.

These results can be explained by hypothesizing the diploid or polyploid nature of the natural hybrid. Changes in ploidy are commonly reported for hybrids in other groups of organisms as well as in fungi (Kuldau 1999). While further studies are needed to determine the exact ploidy of hybrids, it should be noted that we could not differentiate the size of DAPI-stained nuclei between the hybrid and the two other isolates. This may indicate that hybrid nuclei may be diploid, rather than polyploid. As ploidy increases over 2, a significant and detectable increase in nuclear size should be evident.

Pathogenicity and virulence of hybrids assessed through inoculation trials

In experiment 1 and 2 (Fig. 1), the P isolate killed significantly more pines than the S isolate (ANOVA $P<0.001$). However, no significantly different levels of white fir mortality were detected among different ISGs. Furthermore, the hybrid caused as much mortality as the S and P isolates ($P=0.5$) on the universal host, Sitka spruce, indicating its pathogenic potential when the mechanisms of host-specificity are absent.

It should be noted that hybrids from experiments 1 and 2 were characterized by the presence of an intron, that has been associated with the mitochondrial genome of the S ISG (Garbelotto 1996). Our results confirm that virulence on pines is correlated with mitochondrial type (Stenlid 2001), but further indicate the fitness of hybrids by showing they can be virulent on additional species.

We also found that the SP hybrid colonized the inoculated roots at the same rate as that of S or P isolates ($P=0.6$, Fig. 2) and HPLC profiles of SP-inoculated roots were indistinguishable from those of S- and P-inoculated roots (data not shown). This demonstrates that bypassing the host's specific defense barriers can effectively remove the fitness handicap of a hybrid.

Potential for hybridization: the ecological and geographic picture

In California, large-scale logging is a relatively recent event, beginning in the late 1800s. Stumps, an obvious by-product of logging, are ideal colonization courts for *Heterobasidion* spores (Rishbeth 1952; Otrosina 1989). Only 7-17% of the isolates from standing pines and junipers were S. The percentage of S isolates in stumps ranged between 25 and 91%. The increase of the S ISG in stumps ranged from twofold (Modoc NF) to twelve-fold (Inyo NF) (Fig. 2a). Even in the Modoc, the proportion of S isolates in stumps was significantly higher than that in trees (stump S mean=0.3, n=5, SD=0.13; tree S mean=0.07, n=5, SD=0.1. Student's t=3.23, DF=8, $P=0.012$). Both ISGs can be isolated from the same stumps in these areas.

In all NFs, air-borne spores displayed a proportion of genotypes significantly skewed in favor of the S ISG (χ^2 test, $P<0.0001$, df=2). The proportions of S isolates from stumps and the air-spora were virtually indistinguishable in the Inyo and Eastern Plumas NFs (χ^2 test, $P=0.46$, df=1; Fig. 2a), suggesting that the availability of human-made stumps is driving the present-day genetic structure of *Heterobasidion* populations in these forests.

Under the assumption that a 1:1 ratio of isolates of the two species provides the most favorable condition for hybridization, it would be predicted that the Modoc NF is the area most conducive to hybridization (Fig. 2a). It is interesting to note that: a) the natural hybrid was found in this NF, and that b) this NF is characterized by the highest level of introgressed alleles (data not shown).

In California, there appears to be a clear geographic/ecological partitioning in the distribution of *Heterobasidion* species. In mesic mixed conifer sites there is an overwhelmingly dominance of the S ISG. This dominance decreases as we move further into drier pine-juniper sites. Only the P ISG is retrieved from pine stumps in areas where no significant true fir/hemlock populations are in the vicinity (Fig. 2b).

While spatial isolation between stumps and sources of S inoculum (i.e. S host tree species) would theoretically slow down the shift in population structure of this pathogen, the practice of fire suppression over the last 50 years has enabled the encroachment of true firs (S host) in pine/juniper forests (Rundel 1977), progressively reducing such geographic barrier to *Heterobasidion* hybridization.

The combined effects of logging and fire suppression in California have significantly altered the population structure of *Heterobasidion*, and potentially increased the chances for episodic selection (Brasier 1995) and novel evolutionary developments. The effects may be long reaching, as root pathogens not only cause tree mortality but also influence forest composition and succession (Holah 1993).

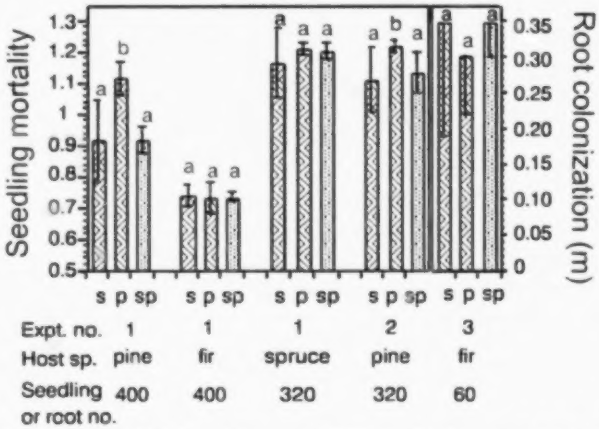
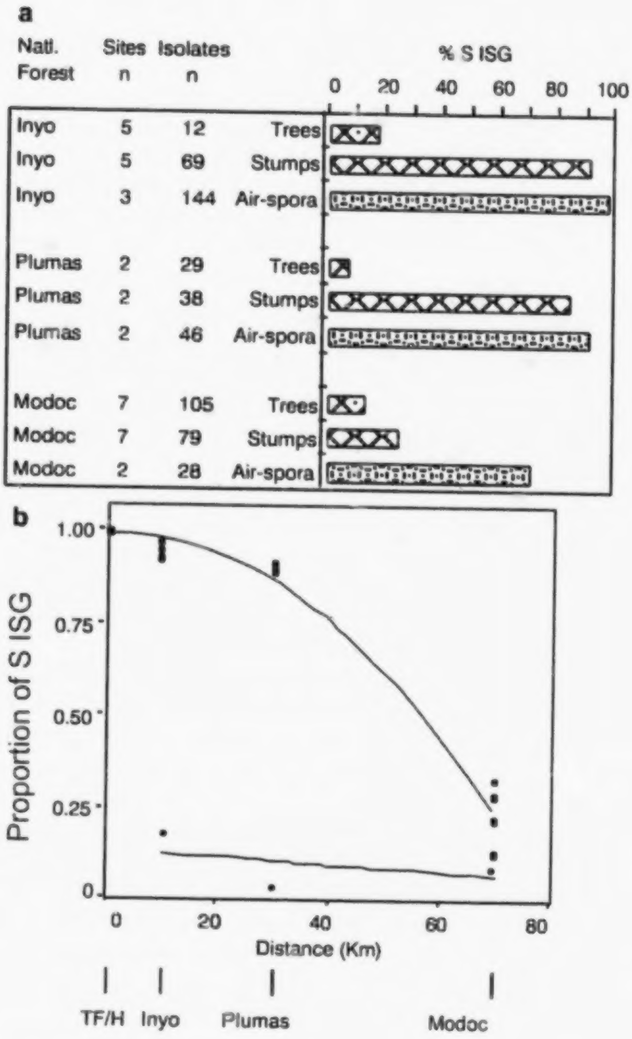


Figure 1. Results from three inoculation experiments: experiments 1 and 2 were onto seedlings in the greenhouse at U.C. Berkeley, and experiment 3 was into roots of white fir trees at Blodgett Forest (Eldorado NF). Seedlings were inoculated in completely randomized blocks with *Heterobasidion* isolates and mortality was scored cumulatively in a 12-month period (Worrall, 1983 #29). On the Y axis, mortality is expressed as $\sqrt{(0.5 + \text{proportional seedling mortality})}$. In experiment 3, 60 roots were inoculated in four randomized blocks as described in Garbelotto et al. (Garbelotto 1997). Extent of fungal colonization in the inoculated roots was measured after six months (data shown with one SD). ANOVA and Tukey Kramer HSD multiple range tests were performed separately for each species (letters indicate homogeneous groups at alpha=0.05).

Figure 2.



(a) Proportions of *Heterobasidion* ISGs from trees (T), stumps (S), and air-spora (A). Pooled data from all study sites within each NF are presented, however distributions were compared either with t-tests using individual sites as replicates, or alternatively with χ^2 tests (H^o= % S ISG in stumps= % S ISG in trees= % S ISG in air-spora).

(b) Results of regression analyses correlating the proportion of S isolates in stumps (filled squares) and trees (filled circles) and the distance from stands with a co-dominance of true fir or hemlocks (TF/H) for at least two miles. Data were from four TF/H sites (only stumps), three sites in the Inyo NF, two sites in the Plumas NF, four sites in the Modoc NF, and two sites in the Cleveland National Forest (only stumps). Regression analyses showed a statistically significant positive correlation ($Y=1.05-0.011X$, $R^2=0.94$, $P<0.0001$) between proportion of S isolates from stumps and proximity to TF/H stands, but no significant correlation was found for S isolates from trees ($R^2=0.056$).

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AIR-BORNE INOCULUM COMPOSITION, PATTERNS OF INTER-GROUP GENE FLOW, OF *HETEROBASIDION ANNOSUM* COLL. SPECIES IN PURE AND MIXED NATURAL FORESTS IN THE ALPS

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SUMMARY

Two natural pure fir and one pure spruce forests showing a stable tree host composition and a mixed dynamic spruce-fir forest (whose tree species composition is changing rapidly) were sampled in the Northwestern Italian Alps. Using woody spore traps, more than 500 spores of *Heterobasidion annosum* coll. were isolated and typed by the Taxon Specific Competitive Priming (TSCP)-PCR combined with a PCR-mediated detection of species-specific introns in the ML5-ML6 DNA region of the mt LrRNA. The three *H. annosum* species co-occurred in most of the sampled forests. Strong correlations were observed in the pure forests between *H. abietinum* and fir (about 80% and 96% of *H. abietinum* spores vs 95% and 100% fir respectively) and between *H. parviporum* and spruce (99% of *H. parviporum* spores vs 100% spruce). In the mixed dynamic forest, no clear relationship between preferential hosts and host-specialized *H. annosum* species was found (i.e. 11% of *H. abietinum* spores vs 50% fir), probably because of the recent establishment of fir at this site. Strong host-specificity of *H. annosum* species in adjacent forests (i.e. 3 km from each other) that greatly differ in tree host composition suggests a restricted dispersal range of *H. annosum* airborne inoculum. Using PCR-RFLP markers on ITS, no nuclear-mitochondrial chimeras between *H. parviporum* and *H. annosum sensu stricto* were detected. This suggests limited gene flow between these two species.

Our results indicate that the knowledge of past and present silvicultural practices is essential in understanding and predicting the population structure of this pathogen.

Keywords: *Heterobasidion annosum*, host specificity, ISGs, gene flow, Alps

INTRODUCTION

The complex *Heterobasidion annosum* (Fr.) Bref. includes at least five allopatrically and sympatrically differentiated intersterility groups (ISGs) (Otrosina et al. 1993): the S, F and P ISGs in Eurasia and the S and P groups in North America (Korhonen 1978; Chase and Ullrich 1988; Capretti et al. 1990). ISGs have recently raised to the rank of species with the following names: *H. parviporum* Niemelä and Korhonen (ISG S), *H. abietinum* Niemelä and Korhonen (ISG F) and *H. annosum* (Fr.) Bref. *sensu stricto* (ISG P) (Niemelä and Korhonen 1998).

Intersterility barriers in a collective species are often associated with the development of host specificity or preference (Burnett 1983). In Europe, *H. annosum sensu stricto* (s.s.) is typically associated with mortality of trees in the genus *Pinus* (mostly of *P. sylvestris* trees), while *H. parviporum* and *H. abietinum* are principally associated to spruce (*Picea abies* (L.) Karsten) and fir (*Abies alba* Miller), respectively.

The European distribution of the three fungal species reflects that of the forests of their native main hosts (Korhonen et al. 1998). Where the host ranges overlap (i.e. in the Alps), all three species can be found (Barzanti and Capretti 1997, La Porta 1999). However, little is known about the degree of host specificity of each species in individual natural forests. The issue of host specificity is complex because often species of *H. annosum* can be found on hosts that are not considered to be the preferential ones and in general it would be more appropriate to use the term host preference rather than host specificity in the case of the *H. annosum* complex (Korhonen et al. 1992; Capretti et al. 1994; Korhonen and Piri 1994; Barzanti and Capretti 1997; Vasiliauskas and Stenlid 1998; Korhonen et al. 1998).

Disease development is also related to site history. Sometimes, *H. annosum* species may be found in stands where their preferred hosts are absent and this is in general attributed to the presence of such hosts in previous rotations and to the adaptation of a species to a secondary host (Korhonen et al. 1998). This finding underlines the fact that forests should not be considered as stable coenoses since they are subject to slow and continuous evolutionary processes. There is still limited information on how forest dynamic processes may affect the population structure of *H. annosum* complex.

Although *in vitro* experiments have shown that in *H. annosum* coll. intersterility barriers are not complete (Chase and Ullrich 1990; Korhonen et al. 1992), in nature sexual incompatibility seems to be quite strict (Otrosina et al. 1992; Stenlid et al. 1994). Several studies have shown absence of inter-group gene flow in Europe (Stenlid and Karlsson 1991; Garbelotto et al. 1998). Hybrids among species (e.g. S-P hybrids) have been found once only in North America (Garbelotto et al. 1996b).

The goals of this study in the Northwestern Alps were: (*i*) to investigate the presence and the degree of host specificity of *H. annosum* species in stable naturally regenerated pure spruce and fir forests (i.e. forests with a stable tree host composition), by comparing relative frequencies of hosts and air-borne spores of host-specific species; (*ii*) to compare spore frequency of each species in forests that are adjacent to one another but differ in tree composition; (*iii*) to assess the relationship between host composition and species composition of the pathogen in a mixed dynamic spruce-fir forest (i.e. a forest whose tree species composition is changing rapidly); and (*iv*) to study the potential for gene flow among species present in the same forest by searching for fungal genotypes bearing the nucleus of one species and the mitochondrial genome of another species.

MATERIALS AND METHODS

Study sites and sample collection

The experiments were performed in 1998-1999 in four natural forests. A pure fir forest in Chiusa Pesio (CN) (100% fir), a forest with about 95% of fir and 5% of spruce (Jovençan, AO), a pure spruce forest (Charvensod, AO) (100% spruce), and a mixed fir-spruce dynamic coenosis (Aymavilles, AO) (50% fir, 40% spruce, 10% Scots pine) were selected for this study. The last three stands were within 5 km of one another on the same mountain slope.

In each forest, two permanent plots were established approximately 100 m from each other. Each plot was characterized by three converging transects (120° from each other), each including three collection points placed at 5, 12 and 19 m from the center of the plot.

Spores of *H. annosum* coll. were trapped using wood disks of fir and/or spruce according to the wood-disk exposure method (James and Cobb 1984). Sets of open Petri dishes, one dish per exposure point, were placed on the ground or on snow for 24 h, starting at 8 a.m. One collection per season was performed (April, July, October and February) during periods with no rain and no wind. Three closed Petri dishes were included as controls on each collection time. The disks were incubated at about 24°C for 10-12 days. Isolations were made under a dissecting microscope by transferring hyphae of *H. annosum* from distinct colonies onto Petri dishes filled with a PCNB-based selective medium for *H. annosum* (Kuhlman and Hendrix 1962). When three or more colonies per disk were visible, three randomly chosen colonies were isolated. All isolates were subsequently grown at 24°C on 5 cm Petri dishes filled with MEA.

PCR primers and DNA amplification conditions for the mitochondrial typing

DNA extractions were made by the CTAB extraction method described by Gardes and Bruns (1993).

A two step process was used to characterize 582 spores (77 from Chiusa Pesio, 223 from Jovençan, 59 from Charvensod and 223 from Aymavilles). First, to distinguish isolates of *H. parviporum* from isolates of the other two species, a Taxon Specific Competitive Priming (TSCP) PCR (Garbelotto et al. 1996b) was conducted in the ML5-ML6 DNA region of the Mitochondrial Large Ribosomal RNA gene (mt LrRNA) using the primers MLS, MLF and Mito 5 (Garbelotto et al. 1998) (Fig. 1). Since *H. abietinum* can be distinguished from the intronless *H. annosum* s.s. because of the presence of an 1.6 or 1.8 Kbp intron in the same region of the mitochondrial DNA (Garbelotto et al. 1998), the second step was to determine the presence of those specific introns. For this purpose, 2 primers named Mito 7 (5'- GCC AAT TTA TTT TGC TAC C -3') and Mito 8 (5'- GCG GTG TAA TAA AAT CGG -3') were designed based on conserved parts of the exon sequence flanking the intron termini (Fig. 2). Amplifications were performed as described by Garbelotto et al. (1998).

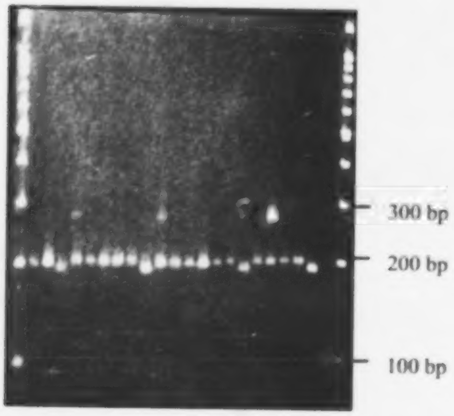

Figure 1. TSCP-PCR in the ML5-ML6 DNA region of the mt LrRNA. Lanes 4, 10, 17 and 22 are *H. parviporum* isolates; the other lanes are either *H. abietinum* or *H. annosum* s.s. isolates. Molecular weight: 100 bp ladder.

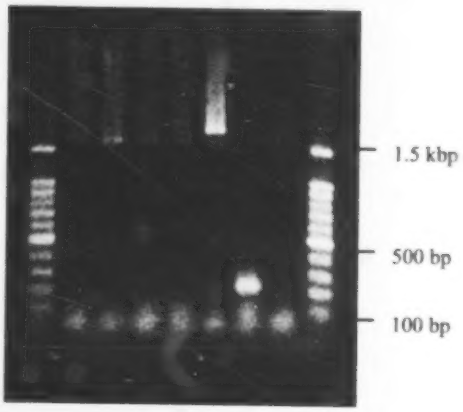

Figure 2. PCR-mediated intron detection of species-specific introns in the ML5-ML6 DNA region of the Mt LrRNA. Lanes 2 to 6 are *H. abietinum* isolates; lane 7 is a *H. annosum* s.s. isolate. Molecular weight: 100 bp ladder.

Amplification products were analyzed by electrophoresis as follows. For the first step they were run in 2.5% Metaphor agarose gels (FMC Bioproducts, Rockland, ME, USA) in 1X Tris-borate buffer (TBE) at 3 V cm^{-1} for 3 hours while for the second step in 1.5% agarose gels (Nu-Sieve, FMC Bioproducts, Rockland, ME, USA.) in 1X Tris-acetate buffer (TAE) at 3.4 V cm^{-1} for 1 hour. Both gels were stained with ethidium bromide.

To confirm the molecular typing, mating tests were performed by pairing 10% of isolates randomly chosen from each plot with homokaryotic testers (T4, T5, T6 - provided by Paolo Capretti - A2r, A27r and A66r) as described by Stenlid and Karlsson (1991).

PCR primers and DNA amplification conditions for the nuclear typing

To check for the occurrence of inter-group hybridization, we searched for the presence of nuclear-mitochondrial chimeras between *H. parviporum* and *H. annosum* s.s. Such chimeras would be expected if hybrids containing nuclei of both species and the mitochondrial genome of one parent were to complete their sexual cycle and produce meiospores. This analysis was performed by characterizing all isolates of the two species both from mitochondrial and nuclear markers, and by comparing results of the two analyses.

The complete ITS region was amplified with the primer combination ITS 1F-ITS 4. Amplification conditions were as above. ITS amplicons were digested with the restriction enzyme *Ban*II (Roche Molecular Biochemicals, Indianapolis, IN, USA) and loaded onto a 1.8% agarose gel, electrophoresed in 0.5X TAE at 3.4 V cm^{-1} for 1 h 20 min and stained with ethidium bromide as described above. While there are no *Ban*II restriction sites in the ITS region of *H. annosum* s.s. isolates, there is one restriction site in the ITS of *H. parviporum* isolates, resulting in two fragments sized 180 and 510 bp approximately (Fig. 3).

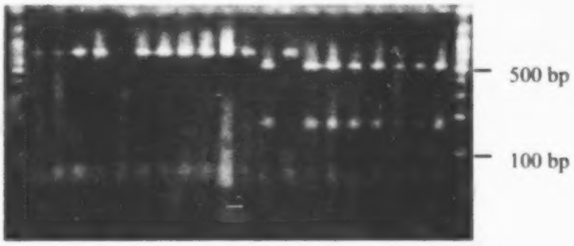

Figure 3. PCR RFLP in the ITS 1F – ITS 4 DNA region of the nuclear genome. Lanes 2 to 11 and 13 are *H. annosum* s.s. isolates; lanes 12 and 14 to 20 are *H. parviporum* isolates. Molecular weight: 100 bp ladder.

Data interpretation and statistical analysis

The frequency of the three species in each study site was expressed as the percentage of spores of each species of the total number of spores collected. Data were used cumulatively for the four collection times to best express the yearly potential of each species to sporulate.

Statistics for the comparison among frequencies of the species within and, for either species, among the study sites were performed by transect and compared by ANOVA - Tukey HSD test, using $P= 0.05$ and $P= 0.01$. The correlation between frequencies of hosts and host-specific species for the evaluation of host specificity in stable and dynamic forests was assessed by the pairwise correlation test. Statistical analysis were performed using STATISTICA and JMP IN.

RESULTS

Airborne spores of *H. annosum* were found at all four collection times in Chiusa Pesio, while no spores were trapped in February in the three sites in the Aosta Valley. The highest inoculum density was recorded in the sampling of July in all the forests. No *H. annosum* colonies were observed on control disks.

In three of the four study sites all three European *H. annosum* species were found. In pure and almost pure fir forests the frequency of *H. abietinum* ranged from about 80% up to 96% (Table 1). Where fir represents 100% of host species susceptible to *Heterobasidion* (Chiusa Pesio), *H. parviporum* and *H. annosum* s.s. had a combined frequency lower than 5%. In fir forests where spruce is sporadically present (Jovençan), *H. parviporum* and *H. annosum* s.s. frequencies were 8% and 13% respectively. In the pure spruce forest, about 99% of the spores were typed as *H. parviporum* spores, and no *H. abietinum* spores were trapped.

Table 1. Northwestern Alps. Frequency of species of *Heterobasidion annosum* coll. in pure stable fir and spruce forests and in a mixed dynamic spruce-fir forest. Within each forest, frequency values of the three species are compared by ANOVA – Tukey HSD test ($P \leq 0.01$).

Forests	Host	%	*H. abietinum* %	*H. parviporum* %	*H. annosum* s.s. %
Chiusa Pesio	Fir	100	95.7 ± 4.4 b	0.6 ± 0.8 a	3.7 ± 4.5 a
Jovençan	Fir Spruce	95 5	79.0 ± 3.1 b	8.3 ± 2.9 a	12.7 ± 3.3 a
Charvensod	Spruce	100	-	98.5 ± 1.5 b	1.5 ± 1.5 a
Aymavilles	Fir Spruce Scots pine	50 40 10	11.2 ± 3.3 a	63.6 ± 2.7 b	25.2 ± 2.7 a

Statistics for the comparison between frequencies of host and host-specific species in each forest and globally between stable and dynamic forests are given in Table 2. In the completely pure forests, significant positive correlation ($P < 0.05$) between *H. annosum* species and preferential host frequencies were found. In the almost pure fir forest in Jovençan the correlation was significant at $P < 0.10$. In the mixed dynamic forest no significant relationship between tree host species and host-specialized species was found. In spite of a relative dominance of fir, *H. abietinum* represented only 11% of the total number of spores collected (Table 1).

Table 2. Statistical analysis for the assessment of the correlation between relative frequencies of hosts and host-specific species of *Heterobasidion annosum* in stable and dynamic forests.

Forests	Pairwise correlation test		
	Value	count (n)	P
Chiusa Pesio	0.9996	3	0.018
Jovençan	0.9946	3	0.066
Charvensod	0.9999	3	0.008
stable forests	0.9922	9	0.001
Aymavilles dynamic forest	0.0194	3	0.9877

Frequency values of *H. parviporum* and *H. abietinum* differed significantly among adjacent forests in Aosta Valley ($P < 0.01$) while the frequency of *H. annosum* s.s. spores showed significant differences only in the comparison between Charvensod and Aymavilles. *Heterobasidion annosum* s.s. was the only species present in all the sampled forests and its frequency was low both in pure spruce and fir coenoses, while it increased significantly ($P < 0.05$) in the mixed dynamic forest (Aymavilles), where Scots pine trees are present.

Nuclear PCR-RFLP typing on 243 *H. pariviporum* and *H. annosum* s.s. spores indicated the absence of nuclear-mitochondrial chimeres. In 100% of the cases, nuclear typing confirmed the mitochondrial characterization. Sexual tests carried out on the subset of isolates fully confirmed the molecular typing.

DISCUSSION

All three European species of *Heterobasidion annosum* coll. have been found in a few countries of Central and Southern Europe (Korhonen et al. 1998; La Porta et al. 1998), including parts of the Alpine range (Barzanti and Capretti 1997). Recently, the first case of co-occurence of all European species in a very large planted stand encompassing different forest types has been reported for the Eastern Alps (La Porta 1999). In our study, all three species have been found within a few square meters in the same forest. To our knowledge, this is the first evidence of co-occurrence of all species in natural coenoses.

In stable pure spruce and fir forests, host preference of *H. parviporum* and *H. abietinum* was very strict, as exemplified by the observation that at least 96% of the trapped spores belonged to a species whose preferred host was present in each forest. Our results, obtained with a novel and systematic sampling approach, are mostly in accordance with those obtained by direct sampling (Korhonen et al. 1992; Capretti et al. 1994; La Porta et al. 1998).

Low frequencies of non specific species (e.g. *H. parviporum* and *H. annosum* s.s.) in the pure fir forest of Chiusa Pesio, where the host appears to be susceptible only to *H. abietinum*, can be due to spores belonging from basidiomes developed in the same forest on hosts which are not the common ones (i.e. white fir and/or deciduous species). These low frequencies can also be interpreted as background contamination due to spores coming, as they can do (Stenlid 1994), from other forests. It should be noted that such background levels are very low and do not appear to significantly contribute to the make up of local *Heterobasidion* populations. Although the frequency of *H. annosum* s.s. is also low in Jovencan (13%) and Charvensod (1%), in these two locations the presence of such a species can be directly linked to the presence of spruce, a well-documented alternate *H. annosum* s.s. host (Korhonen et al. 1992; Korhonen and Piri 1994; Vasiliauskas and Stenlid 1998). Direct stump sampling in Charvensod (data not shown), has confirmed the significant presence of *H. annosum* s.s. isolates in spruce stumps at that site.

Pure and almost pure fir forests harbor an overwhelming majority of *H. abietinum* inoculum, and the same is true for *H. parviporum* inoculum in the pure spruce forest. In our study this strong host-pathogen correlation held true even for two forests (Charvensod and Jovençan) different in tree composition, but only 3 km from each other. The apparent lack of spore flow between these two nearby sites suggest a geographically limited range of dispersal of basidiospores. This information is important from both an ecological and an epidemiological point of view and supports the belief that during thinning operations, the presence of basidiospore-producing fruit-bodies increases the risk of stump infection within but not between forests (Stenlid 1994; Möykkynen et al. 1997).

In the mixed fir-spruce dynamic forest there was no direct correlation between frequency of *H. abietinum*/*H. parviporum* and frequency of their respective preferential hosts. In that forest, lack of thinning operations since the 1940s has lead to the progressive and massive establishment of firs in a spruce stand. It appears that a corresponding "invasive" establishment of *H. abietinum* (11% of all trapped spores) is following the "invasive" establishment of the host species. Primary infection and spread of *H. annosum* occurs by the means of airborne spores (Rishbeth 1951; Stenlid 1994). Large fruit body production and the subsequent massive release of airspora normally occur after colonization of large volumes of substrate. Furthermore, substrate colonization leading to fruit-body production may require several years and may be more likely to occur on larger trees. We expect that as fir trees grow larger and the overall fir biomass increases, so will the population of *H. abietinum*.

It has been suggested that the biogeographical history of the main hosts of *H. annosum* may have influenced the genetic structure (Stenlid et al. 1994) and the present distribution of the three species (Korhonen and Piri 1994). Our results indicate that, within a shorter time frame, rapid changes in host composition and past and present silvicultural practices may have an effect on the population structure of this pathogen.

No nuclear-mitochondrial chimeras between *H. parviporum* and *H. annosum* s.s. were found in this study. Lack of hybridization and potential for gene flow among *H. annosum* species in Europe has already been discussed (Stenlid and Karlsson 1991; Garbelotto et al. 1998). Generally, fungi from the same geographical area exhibit strong genetic barriers to interspecific hybridization (Brasier 2000). The potential for hybridization will also depend upon the frequency of niche contact and the ability of any resulting hybrids to compete with the parent species. In the Western Alps different niches are in close proximity. In this study we show that spruce, fir, and Scots pine and the three host-associated *H. annosum* species are co-existing. The presence of both *H. parviporum* and *H. annosum* s.s. isolates on spruce, and the creation of non-selective stumps through logging are examples of true niche overlaps and increase the chance for hybridization (Garbelotto et al. 1996b; Garbelotto et al. 1998). Lack of hybridization between *H. parviporum* and *H. annosum* s.s. in the Alps may be determined by efficient mating barriers between the two populations reinforced by negative selection on hybrids, as reported for the American groups (Garbelotto et al. 1996a).

Results showing strong correspondence between hosts and host-associated *H. annosum* species in individual forests and lack of significant spore movement between adjacent coenoses have important implications in forest planning and management. This information, in fact, can be used to determine the usefulness of tree species substitution in areas infested by this pathogen.

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ISOLATION AND MOLECULAR STRUCTURE OF A LACCASE GENE FROM THE ROOT ROT FUNGUS *HETEROBASIDION ANNOSUM* (S-TYPE)

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SUMMARY

Using a synthetic olignucleotide primer, a cDNA and genomic DNA coding for laccase of the conifer pathogen *Heterobasidion annosum* was isolated by PCR amplification, cloned and characterised. Nucleotide sequence determination of the *ca* 1.64kb PCR fragment revealed an open reading frame encoding a polypeptide of 330 amino acid residues. Putative exons deduced on the basis of homology to other fungal laccases and analyses of the sequence revealed that about nine small introns interrupted the genomic DNA. Comparative analyses of the predicted amino acid sequence showed great similarity to the laccases from *Pleurotus ostreatus*, *Phlebiopsis radiata* and *Trametes versicolor*.

Keywords: DNA, *Heterobasidion annosum*, introns, laccase, PCR

INTRODUCTION

Root and butt rot of conifers cause annual economic losses of 500 -1000 million crowns in Sweden and it is the most important conifer disease in a global perspective. The main causative agent, *Heterobasidion annosum* (Fr.) Bref., is a white rot fungus that degrades both lignin and cellulose (Asiegbu et al. 1998, Daniel et al. 1998). Whereas most wood decayers are saprophytes, *H. annosum* is a strong parasite, able to infect and destroy living conifer roots and stems of all ages (Asiegbu et al. 1998). The fungus is spread by basidiospores to stumps and wounds and by mycelia via root contacts from tree to tree (Hodges 1969, Stenlid 1985). The fungus is known to comprise three intersterile subspecies in Europe: S, P and F. These groups show pathogenic specialization on wood species of spruce (i.e. S and P), pine (i.e. P) or silver fir (*Abies alba*) (i.e. F) (Korhonen 1978, Capretti et al. 1990).

Both saprotrophs and necrotrophic parasites like *H. annosum* secrete extracellular enzymes which can degrade the cell wall components of susceptible woody plants (Daniel et al. 1998). These fungi digest plant cell wall polymers not only for the supply of nutrients but also to penetrate cells and spread through woody tissues. Among several enzymes secreted by *H. annosum* during the interaction process, pectinases (Johansson 1988, Karlsson and Stenlid 1991) have been widely studied, but not much is known about laccases which presumably can contribute substantially to lignin degradation and to detoxification of host chemical and structural defences (Haars and Huttermann 1980, Johansson et al. 1998). As copper containing enzymes, laccases are also known to be involved in different biological processes such as sporulation, pigment production and fruit body development (Leatham and Stahman 1981). According to Coll et al. (1993), detailed studies of the structure and regulation of the laccase coding genes would help in the characterization of the roles and enzymatic mechanisms of the different laccases in specific physiological processes.

To date, only few reports are available on laccase sequences from white rot and non lignolytic fungi (Germann et al. 1988, Aramayo and Timberlake 1990, Kojima et al. 1990, Saloheimo et al. 1991, Choi et al. 1992, Perry et al. 1993, Coll et al. 1993). However, none of the laccases of necrotrophic fungal pathogens of conifers such as *Heterobasidion* sp. have been sequenced. This study therefore is the first on the isolation and characterization of partial sequence structure of *H. annosum* laccases.

MATERIALS AND METHODS

Micro-organisms

Escherichia coli, DH5α-competent cells were purchased from Life Technologies, Sweden. Homokaryotic strain of *Heterobasidion annosum*, S-type (FSE-7) was obtained by courtesy of Prof. Jan Stenlid (Dept. Forest Mycology and Pathology, Univ. of Agriculture, Uppsala, Sweden).

Chemicals

Chemicals used as buffers and substrates were of at least reagent grade.

Genomic DNA isolation

Cultures of *H. annosum* (FSE-7) were grown in 500 ml of Hagem medium (Stenlid 1985) at room temperature for 6 - 8 weeks. Mycelia were harvested by filtration through Whatman No.1 filter paper, washed twice with sterile distilled water and frozen quickly in liquid nitrogen. The frozen mycelia was ground in a mortar, transferred to sterile tubes followed by addition of extraction buffer (2% cetyl trimethyl ammonium bromide (CTAB), 100mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.2% mercaptoethanol). The mixture was incubated at 68°C for 50 minutes, followed by the addition of equal volume of chloroform : isoamyl alcohol (24:1), vortexed and centrifuged at 12,000 x g for 5 min at 25°C. About 1.4 volume of isopropanol was added to the upper aqueous phase in a fresh tube and left to stand at room temperature for 10 min to precipitate the nucleic acid. The precipitate was recovered by centrifugation and the pellet resuspended in TE (10 mM Tris-HCl pH 8.0, I mM EDTA), the RNA was removed following digestion with 3 to 5 units of RNase at 37°C for 1 hr and the mixture emulsified by adding an equal volume of chloroform: isoamyl alcohol (IAA), vortexed, and centrifuged at 12000 x g for 5 min at 25°C. The aqueous phase was transferred to a fresh tube and the DNA precipitated by addition of one tenth volume of 3M Na - acetate and 1.5 volume of cold absolute ethanol. The pelleted DNA was recovered by centrifugation at 12, 000 x g for 3 min at 4°C, the supernatant was discarded and the later precipitation steps were repeated once before the pellet was finally resuspended in TE.

RNA preparation

RNA was isolated from mycelia that were harvested from *H. annosum* cultures which either had been induced for laccase expression by addition of ferulic acid (Daniel et al. 1998; Johansson et al. 1998a, b) or were uninduced. Ferulic acid was added to a final concentration of 0.002% and the cultures were allowed to grow for 3 to 4 weeks. The mycelia were washed and frozen quickly in liquid nitrogen. RNA was isolated from the mycelia using a modified procedure of Chang, Puryear and Cairney (1993). Briefly, mycelia were ground in liquid nitrogen, added to extraction buffer (2% CTAB, 2% polyvinyl pyrollidone (PVP), 100 mM Tris HCl pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5g/l spermidine, 2% B-mercaptoethanol) and shaken vigorously, and left in a water bath at 65°C for 45 min. After two extractions with an equal volume of chloroform: IAA (24:1), a one-quarter volume of 10 M LiCl was added and the RNA was precipitated overnight at +4°C. The pellet was dissolved in SSTE (1.0M NaCl, 0.5% SDS, 10 mM Tris HCl pH 8.0, 1mM EDTA pH8.0), extracted once more with chloroform : IAA and reprecipitated with two volumes of ethanol at -70°C for 2 hr. The RNA pellet was dried and dissolved in DEPC treated water. PVP was omitted in extraction buffer for cultures not grown in the presence of ferulic acid.

Preparation and PCR amplification of cDNA

Polyadenylated RNA was selected on an oligotex beads according to manufacturers instruction (QIAGEN). First strand cDNA was synthesized with reverse transcriptase (GIBCO BRL) as described in the suppliers instructions (Clonetech). The cDNA was PCR amplified through modification of Clonetech procedure by using gene specific primers (Lac1 3' and Lac1 5', see below).

Oligonucleotide probes

Oligonucleotide sequences were designed based on the conserved, published nucleotide sequence of laccase genes from five related basidiomycetes.

PCRs

For PCR, 100 ng of genomic DNA was mixed with the primers (Lac1 5'; AGC AYT GGC AYG GCT TYT TCC A; Lac1 3'; GTC RAT GTG GCA RTG GAG GAA CCA) under standard PCR conditions (Sambrook et al. 1989) [5 min at 95° (1 cycle), 1 minute at 95°, 1 min at 55°, 2 min at 72° (30 cycles), and 5 min at 72° (1 cycle)].

Cloning

The 1.64 kb genomic laccase DNA was exercised from agarose gel, gene cleaned (BIO 101), cloned by blunt end ligation into pUC18, plasmid vector (pLac:FSE-7) using the SureClone ligation kit according to suppliers instructions (Pharmacia Biotech, Sweden).

Southern blots

DNA samples were digested with either *Hind* III, *BamH* I or *EcoR* I, electrophoresed on agarose gels in TAE (0.04 M Tris acetate, 10 mM EDTA) buffer by standard protocols (Sambrook et al. 1989). The DNA was transferred to a Genscreen plus membrane (DUPONT). Blots were hybridized at 42°C in hybridization solution (50% formamide, 1M NaCl, 10% SDS, 250 µg of denatured salmon sperm DNA per ml) and washed at room temperature in 1% SDS plus 2 x SSC (0.3 M sodium chloride - 0.03 M sodium citrate for 30 minutes and at 65°C for 20 minutes with 1% SDS plus 0.1 x SSC (0.015 M NaCl-0.0015M sodium citrate). Radioactive probes were prepared with (α - ^{32}P)dCTP (DUPONT).

DNA sequencing

Nucleotide sequences were determined by Taq polymerase cycle sequencing with fluorescently labelled nucleotides and the reaction mixtures were run on an Applied Biosystems automatic DNA sequencer (model 377, version 3.2). The DNA sequences were translated into predicted amino acid sequence by using Grail exon computer programme. Amino acid sequences were compared with the sequences available in the EMBL and Genebank databases by using the FASTA and BLAST search programmes, respectively. Alignment of the nucleotide sequences was carried out with the CLUSTAL program.

Nucleotide sequence accession number

The sequence of the *Heterobasidion annosum*, S-type laccase genes reported in this paper has been assigned EMBL accession no. Y16951.

RESULTS AND DISCUSSION

Production of laccase activity by *H. annosum* in batch cultures (Johansson et al. 1998 a, b) similar to that used for the RNA isolations has earlier been described. Some laccase mRNA are transcribed 7 days after growth on Hagem medium, but a further increased stimulation of laccase enzyme activity is observed with addition of ferulic acid or 4% spruce wood extract to growth medium. Consequently, ferulic acid was added to culture media to obtain increased levels of mRNA transcription. Synthesis of laccase transcripts were detected for the gene by PCR amplification of cDNA prepared from either mRNA or total RNA using gene specific primers (Fig. 1).

High quality chromosomal DNA amenable to restriction enzyme digestion was isolated from *H. annosum* by slight modification of the previously described method of Zolan and Pukkila (1986). Using the synthetic oligonucleotide primer, a 1.64 kb fragment (Fig. 1) was amplified from the genomic DNA under standard PCR conditions. The PCR gene product was cloned, pLac-FSE-7 by blunt end ligation (Maniatis et al. 1985). Digested genomic DNA from the homokaryotic *H. annosum* (S-type) was hybridized with the PCR product as a probe. The single band on the Southern autoradiograph suggested the presence of a single laccase gene in the homokaryotic *H. annosum*, (strain-FSE-7) which also confirmed the presence of the gene in the chromosomal DNA of the fungus (Fig. 2). By contrast, sequence analyses of multiple bands on the PCR products isolated from the genome of homokaryotic *H. annosum*, P-type (Sä 16-4) strongly suggest that it contains at least two different laccase genes (Asiegbu, unpublished).

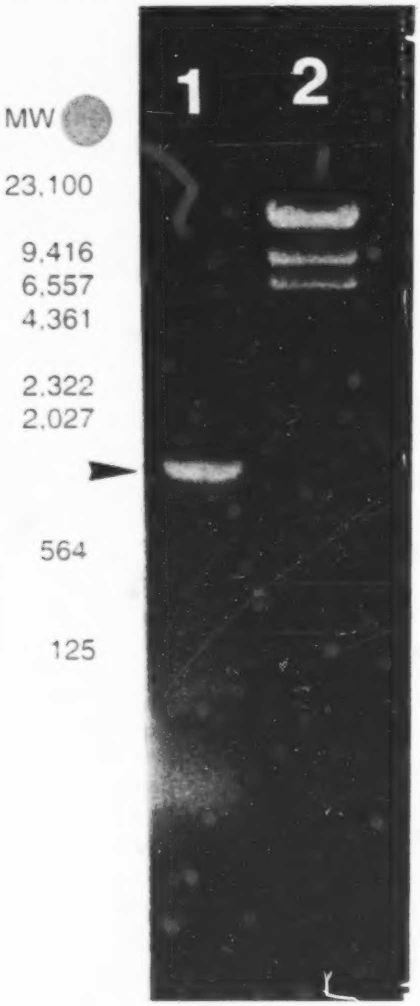

Figure 1. Amplified PCR products (arrowheads) from genome of *H. annosum*. 1.6 kb fragment from S-type (lane 1), lambda-*Hind* III base-pair ladder (lane 2).

Direct sequencing of the 1.64 kb PCR product from S-type by ABI prism revealed a sequence of 1639 bp (see Genbank Accession no. Y16951). Putative intron and exon positions were deduced on the basis of homology to other fungal laccase and by sequence analyses of the *ca.* 1.0 kb PCR amplified cDNA product. The laccase gene isolated from the cDNA and genome encodes a predicted protein of *ca* 330 amino acids respectively. The coding region is interrupted by nine putative introns. The deduced amino acid sequences of *H. annosum* laccase predict proteins with the structural characteristics of blue copper oxidases (Thurston 1994).

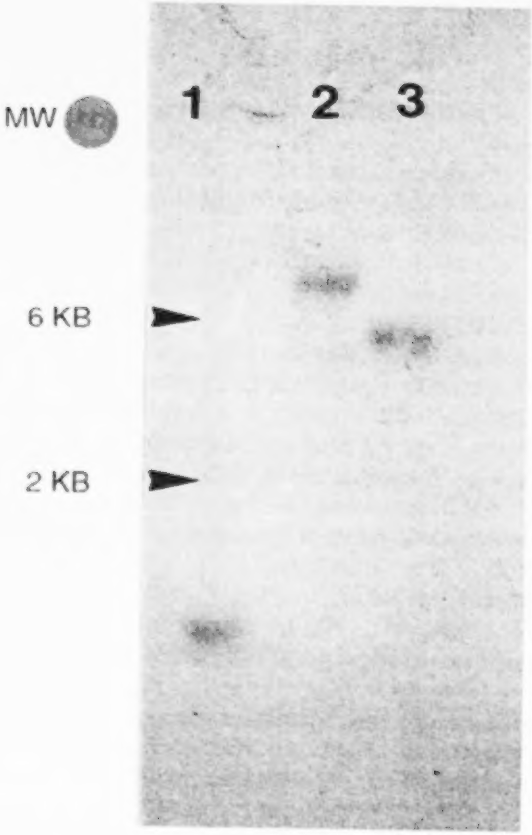

Figure 2. Southern bloting of genomic DNA from S-type of *H. annosum* using a radioactive labelled laccase PCR product as a probe showing the presence of laccase gene in the genome. The genomic DNA was digested with restriction enzymes *BamH* I (lane 1), *EcoR1* (lane 2) and *Hind* III (lane 3).

Comparison of the deduced amino acid sequences of the laccase gene from *H. annosum* with other multicopper blue proteins shows significant homology in one of the four copper binding regions (Saloheimo et al. 1991). The regions that coordinate Cu^{2+} ions are perfectly conserved. This homologous region is rich in clusters of histidine residues (His-X-His) which constitute the proposed copper binding ligands as shown on the X-ray crystallographic analysis for Cucumis sativis ascorbate oxidase (Ohkawa et al. 1989).

Alignment of the nucleotide sequence of the predicted mature protein coded for by *H. annosum* laccase gene to laccases from other white rot fungi like *Phlebia radiata, Pleurotus ostreatus, Trametes versicolor* and *Agaricus bisporus* show about 69%, 71%, 56%, and 53% identity, respectively. Since the laccase sequences of *H. annosum* can be aligned throughout the whole length of the laccase gene from these fungi, it is possible that the basic architecture of their polypeptide folding is similar. The three repetitive structural domains of laccase and the β-strands as illustrated previously (Saloheimo et al. 1991) as forming the basic fold of *P. radiata* laccase can also be seen in the alignment with *H. annosum* laccase (data not shown). Unlike *P. radiata* laccase, nothing is however known about the structural and biochemical characteristics of the *H. annosum* laccases. Karhunen et al. (1990) suggested that the laccase of *P. radiata* is a quinoprotein in that it contains PQQ as its prosthetic group. PQQ is a two-electron transferring group found in many oxidoreductases and is bound covalently to its apoenzyme in eukaryotic quinoproteins (Duine 1988). Other authors have suggested that PQQ forms an amide bond with lysine residue in the apoprotein of porcine kidney diamine oxidase (PKDAO) (Van der Meer et al. 1989). An alignment of the segment of PKDAO and *P. radiata* laccase sequence (Saloheimo et al. 1991) proposed to carry PQQ with *H. annosum* laccase showed some degree of homology of about 45 and 63%, respectively.

In this paper, we presented the first report on isolation and molecular structure of the laccase gene from the S-type of the conifer pathogen-*H. annosum*.

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ANALYSES OF SELECTED EXPRESSED SEQUENCE TAGS (EST) FROM *HETEROBASIDION ANNOSUM* (P-TYPE) - *PINUS SYLVESTRIS* PATHOSYSTEM

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SUMMARY

Subtractive hybridization technique was applied in order to clone cDNAs representing genes that are differentially expressed during interaction of the necrotroph *Heterobasidion annosum* and its conifer host (*Pinus sylvestris*). Sequence analyses of these cDNAs showed that several kinds of genes were identified such as those involved in disease resistance, signal transduction, metabolism and other cellular functions. The possible roles of these gene products in the interaction are discussed.

Keywords: ESTs, *Heterobasidion annosum, Pinus sylvestris*, root rot, subtractive hybridization

INTRODUCTION

Root rot disease of conifer trees is the most economically important tree disease in the northern hemisphere (Hodges 1969). The disease is caused by the root rot pathogen, *Heterobasidion annosum*. Several conifer species (Norway spruce, Scots pine, silver fir) serve as host to the three forms of *H. annosum* S, P and F-types, respectively. Pre-treatment of stumps with chemicals or the biocontrol agent (*Phlebiopsis gigantea*) are considered the only feasible means of controlling the disease in forest plantations. In recent years, the interest for phenotypic selection for resistance and breeding among recognized resistant trees have increased (Carson and Carson 1989); however, the molecular basis of the host–pathogen interaction in root rot infection are still not fully understood. The root rot fungus (*H. annosum*) which has been used as a model organism to investigate various aspects of the host–pathogen interaction have been intensively studied than the host. Ecological and biochemical factors regulating spore dispersal, germination and host penetration have received much more attention (Redfern and Stelid 1998, Asiegbu et al. 2000) but very few papers have been published regarding genes involved in resistance and pathogenicity such as those that may be differentially expressed during pathogenesis.

However, to obtain an overall view of all the processes that are occurring during fungal invasion, it is necessary to identify as many genes as possible that show induced expression during the interaction. Initial strategy of gene discovery by sequencing of large numbers of cDNAs for identifying expressed genes has only been applied to a few studies (Adams et al. 1991, Tagu and Martin 1995, Cooke et al. 1996). Although large scale ESTs technique has been available since 1991, most of the projects applying this methodology in plant biology has concentrated on identifying genes expressed during developmental processes (Wyrich et al. 1998) and plant responses to environmental cues (Umeda et al. 1994, Liu et al. 1995, Lim et al. 1996, Kwak et al. 1997, Lee et al. 1998). The application of ESTs to characterize genes expressed during symbiotic interaction was pioneered by Tagu and Martin (1995). Similarly, very few papers have been published relating to the application of ESTs to identify genes involved in host–pathogen interactions (Botella et al. 1997).

In this paper we report the use of subtrative hybridization in identifying ESTs of genes differentially expressed during interaction of the conifer tree (Scots pine) with the root rot fungus (*Heterobasidion annosum*).

MATERIALS AND METHODS

Host material and pathogen isolate

Scots pine (*Pinus sylvestris*) seeds were purchased from Assidomain AB, Sweden. *Heterobasidion annosum* (FP5) was obtained from K. Korhonen (Finland) maintained on Hagem agar at 20 ± 1°C. Mycelial of *H. annosum* used for inoculation was obtained from cultures cultivated in liquid Hagem medium for 10 days at static conditions.

Root rot inoculation

Seedlings of *P. silvestris* (16 days old) were transferred to sterile filter paper pre-laid on 1% water agar in Petri dishes. The root regions of the seedlings were inoculated with clumps of mycelia without homogenization (about 0.1 g). For each three seedlings, one clump of mycelium was added together with 0.5 ml of distilled water with a total of 10 seedlings per 90 mm diameter plate. Control seedlings were mock inoculated with sterile distilled water. After inoculation seedlings were exposed to 16 h photoperiod and used for RNA extraction 6 days post inoculation (dpi).

Subtractive hybridization and Library construction

Seedling root materials were used directly after harvest. Total RNA was extracted from 6 dpi and from uninfected Scots pine seedling roots following the procedures of Chang et al. (1993). The cDNA was synthesized using SMART cDNA synthesis kit (Clotech, USA). Excess cDNA from the uninfected seedling roots was used for the subtraction in a two round hybridization and PCR reaction with diluted amounts of adaptor ligated cDNA from the infected seedling roots using the PCR select cDNA subtraction kit (Clontech, USA). During secondary PCR amplification, the background is reduced and differentially expressed genes are further enriched (Clontech, USA). Secondary PCR product from the subtraction was cloned into pT-Adv vector for subtractive cDNA library. Plasmid miniprep DNA of several clones were chosen at random, restricted with *EcoR* I in order to assess the insert size of the cloned cDNA.

Hybridization analysis

Nylon membrane filters were prehybridized at 65°C for 2 hr in 15 ml of hybridization solution (6 × SSC (1 × SSC is 0.15M NaCl, 0.015M sodium citrate), 5 × Denhardts solution (1 × Denhardts solution is 0.02% Ficoll 400, 0.02% PVP, 0.02% BSA), 0.1% SDS and 50 mM phosphate buffer, pH 6.6 at 65°C) with constant agitation. The genomic DNA and cDNA probes were denatured by heating at 100°C for 5 min before adding to the prehybridization solution. Hybridization was performed with gentle agitation overnight at 65°C in a hybridization incubator. After hybridization, membranes were washed with 0.5 × SSC, 0.1% SDS before being exposed to autoradiographic film.

Storage of cDNA clones in microtiter-plates and on nylon membrane filters

The recombinant cDNA clones (12,288) were stored in microtitre plates and also replicated onto hybond N^+ membranes using a Q-bot automated workstation (Genetix, USA) as described by Zhu et al. (1997).

Random sequencing of cDNA from subtractive library and sequence analyses

The plasmid DNA template purification was performed using 96 well format filter plates from 2 ml Terrific broth (TB) cultures. For DNA sequencing, each reaction was performed with 2 µl of Bigdye terminator chemistry (Perkin-Elmer Corp., USA) in a 10 µl reaction with T7-primer using ABI Prism 3700 96-well capillary automated DNA sequencer. Nucleotide sequence and data analysis was performed using a scheme developed at Fungal Genomics laboratory, North Carolina State University, Raleigh. Raw sequence files generated by DNA sequencer were uploaded to a Sun Ultrasparc Unix server and read into Phred and Phrap (Ewing et al. 1998). After deleting vector sequence, cDNA sequences were compared with Genbank database sequences using Blastx. Sequences for which no match was found were classified as unknown. Approximately 500 good sequences were submitted for annotation to Genbank EST database (accession numbers BI416470 – BI416974).

RESULTS AND DISCUSSION

Several methods are available for the investigation of gene expression, subtractive hybridization is however relatively novel and a powerful technique that enables one to obtain clones of genes that are specifically or differentially expressed (Figure 1). In practice, subtractive hybridization is usually applied in order to enrich the target cDNA for fragments that represent genes preferentially expressed in the tissue of interest (Wang and Brown 1991). According to Wang and Brown (1991), this can be achieved by removal of DNA sequences representing genes that are also in the driver tissue. In this case, the target tissue of interest was infected seedling roots of Scots pine and the driver tissue was uninfected seedling roots (control plants). The procedure for the enrichment of target tissue with cDNA specifically induced during infection involves multiple rounds of long hybridization and PCR amplification. The ligation of a specific primer (adaptor) to the target cDNA fragments allows amplification and cloning of fragments derived only from the target cDNA. The total RNA used for subtractive hybridization was isolated from 6 dpi (target issue) and uninfected (driver tissue) Scots pine roots. After subtractive enrichment and amplification, the secondary PCR product was cloned into pT-Adv vector and transformed into *Escherichia coli*. The inserts in the clones were analysed by restriction enzyme digestion followed by gel electrophoresis. The average size of the insert in majority of the clones ranged from 200 – 1800 bp which is within the expected size due to initial digestion of the target cDNA with *Rsa* I and *Alu* I (data not shown).

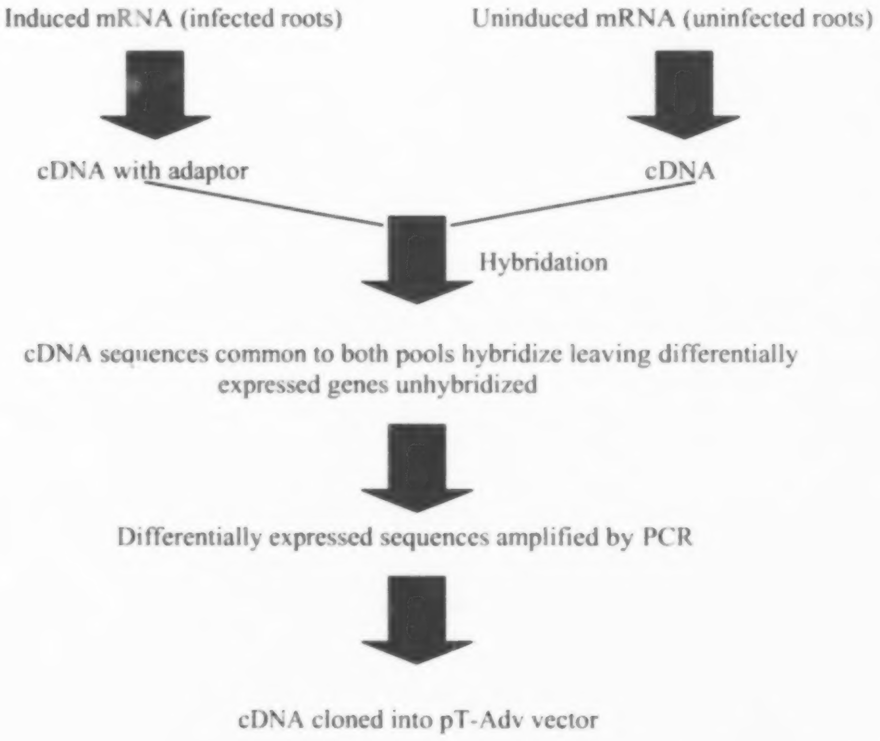
Induced mRNA (infected roots)
Uninduced mRNA (uninfected roots)
cDNA with adaptor
cDNA
Hybridation
cDNA sequences common to both pools hybridize leaving differentially expressed genes unhybridized
Differentially expressed sequences amplified by PCR
cDNA cloned into pT-Adv vector

Figure 1. Subtraction procedure.

About 800 clones from the subtracted cDNA library were randomly selected and partially sequenced. Approximately 202 clones from these sequences were further analysed. Based on Blastx analyses, the most abundantly expressed genes are shown in Table 1 and species groups with homology against Genbank are shown in Figure 2. The largest single group of ESTs (27%) showed similarity to genes involved in cellular functions (Figure 3). The next most prevalent class (17%) showed similarity to defence related proteins representing several gene families (including receptor like protein kinases, antimicrobial peptides (AMPs), Hydroxyprolin rich glycoproteins, genes involved in signal transduction (GTP binding protein, MAP kinases), pathogenesis related proteins, cationic peroxidases, vacuolar ATP-synthase and methallothionin like proteins). About 4% of the ESTs sequences showed homology to photosynthesis related genes (Chlorophyl binding protein etc). A significant percentage of the ESTs (47%) showed similarity to proteins of unknown function or to hypothetical proteins. The remaining ESTs showed no significant match with any sequence in the Genbank.

Table 1. Abundantly expressed host defence related genes.

Homology to	Sequence redundancy
Hydroxyproline rich glycoprotein	9
Antimicrobial peptide (Amp)	9
Signal transduction (Map Kinase, GTP-binding)	4
Cationic peroxidase	2

The abundance of the gene encoding antimicrobial peptide (AMPs) in the subtraction library (Table 1) possibly suggests it's involvement in host–parasite interaction. Consequently this gene (code named Sp-Amp) was used for further study. The Sp-Amp cDNA clone was then used to probe the *H. annosum*–Scots pine cDNA library filter containing about 12,300 clones. The result showed 81 positive clones suggesting that it is highly expressed. Blastx analyses using full length cDNA clone of this gene showed significant homology (e-value of 7e-25) to a similar peptide from *Macadamia integrifolia* (Marcus et al. 1999). Literature reports revealed that the AMP peptide is capable of inhibiting the growth of a variety of fungi and gram positive bacterial phytopathogens (Marcus et al. 1999). Bioinformatic analyses using Phred and Phrap programme revealed the existence of a gene family in Scots pine antimicrobial peptide genes. Four of these genes have been isolated and deposited to the Genebank (accession nos. AF410952, AF410953, AF410954, AF410955) The presence of several disease resistance gene homologues in the present study would tend to support their role in host response reaction during pathogen invasion. Similar disease resistance genes have been identified in other pathosytems such as downey mildew resistance protein (Parker et al. 1997) and wheat leaf rust resistance gene (Feuillet et al. 1997). It is also not surprising that a certain number of the ESTs showed similarity to photosynthesis related genes as mRNA used for the subtraction library were obtained from seedling roots which were cut at the margin interface between root tissues and the beginning of hypocotyl region. Since, Blastx search only matches information to existing sequences in the database, it is therefore not unexpected that about 47% of our ESTs encode genes of unknown function. The value of such sequences, however, should not be under-estimated by being placed under such category. This could merely reflect the fact that the large number of genes induced during host parasite interaction are yet to be fully identified, cloned and sequenced. Consequently, it is not surprising that several of the EST sequences match *A. thaliana* genes which has been thoroughly studied. Several authors have also made similar observations (Cooke et al. 1996, Fristensky et al. 1999). Cooke et al. (1996) showed that only about one third of ESTs from Arabidopsis corresponded to known proteins.

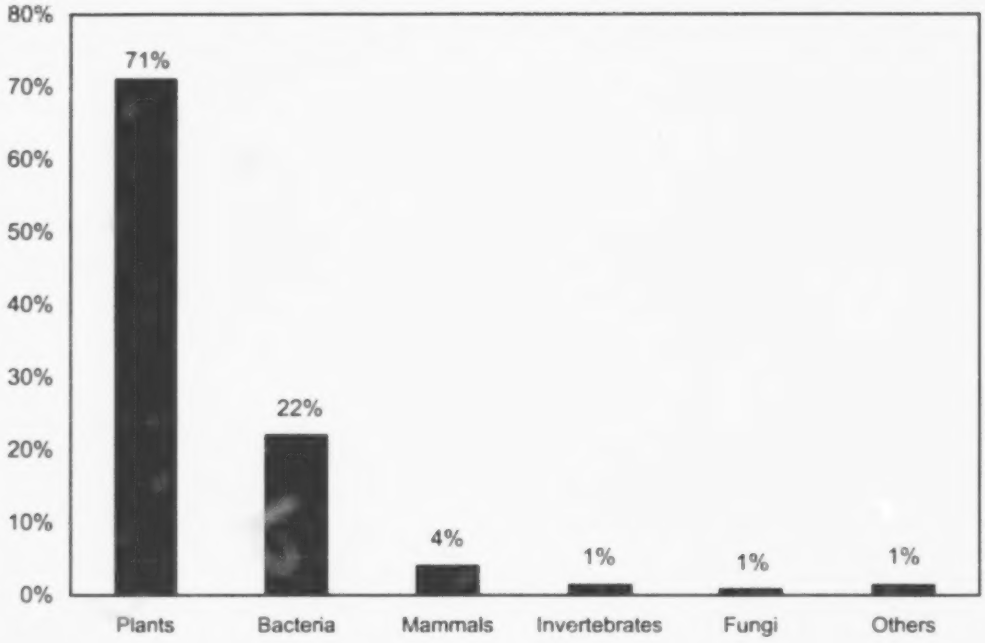

Figure 2. Species groups with homology against Genbank.

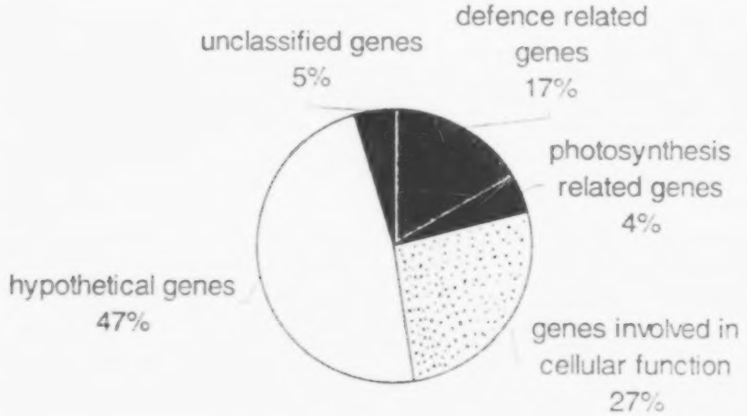

Figure 3. Functional groups in EST's.

The very few fungal genes detected in the subtraction library is consistent with results of other authors (Fristensky et al. 1999). Fristensky et al. (1999) attributed the low number of fungal genes to the fact that the proportion of fungal biomass as compared to plant biomass in the infected tissue is relatively small. This may equally be the case in our own studies since 6 d.p.i. time is considered a short period of time to obtain a high proportion of fungal RNA in a predominantly plant RNA population.

ACKNOWLEDGEMENTS

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EVOLUTION OF *ARMILLARIA* GENETS OVER EIGHT YEARS (1992-2000)

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SUMMARY

The territories of the genotypes ('genets') of *Armillaria* spp. were mapped successively in 1992, 1996 and 2000 on three forest plots of small area, in the French mountain region "Massif Central".

Two plots ('Aix-ouest' and 'Aix-est') are situated in a parcel covered with young Scots pines established from natural regeneration after the clear felling of a mixed forest in 1984, followed by a complete uprooting of the stumps. *Armillaria ostoyae* is the only *Armillaria* species present in this stand; it behaves as a primary parasite on the young pines. The third plot ('Col de Ceyssat') is situated in a 70-year-old silver fir plantation, established in the 1930s after the felling of a natural beech forest. This site harbours both *Armillaria gallica* and *A. ostoyae*; the former species is completely saprophytic while the latter behaves as a weak parasite of fir.

Genet mapping was carried out at Aix-ouest and Aix-est by Somatic Incompatibility (SI) and RAPD; SI appeared slightly more precise than RAPD with four primers. Only SI was used at Col de Ceyssat.

At Aix-ouest, the five genets already present in 1992 were still present in 2000 (however, the area of two of them had been considerably reduced) and no new genet had appeared over 8 years. At Aix-est, the situation of the genets was also very stable in course of time. At Col de Ceyssat, *A. gallica* is represented by two genets, probably older than the fir plantation, which "saturate" the stand with their rhizomorphs and completely overlap. *A. ostoyae* is represented by three small genets, probably younger than the fir plantation, which are expanding in the new stumps by mycelium through root contacts or latent infections.

Although the exact site of the formation of new diploid genets remains unclear, their appearance seems to be a rare occurrence, generally linked to drastic modifications of the forest.

Keywords: Armillaria, population dynamics, biological strategy, somatic incompatibility, RAPD

INTRODUCTION

In the last two decades, the genus *Armillaria* has been the subject of a number of studies in population genetics. Most of these investigations consisted in mapping the natural genotypes ('genets') of *Armillaria* in different types of forests, forest plantations or woody cultures. Since the pioneering work of Korhonen (1978), no less than 26 papers have been devoted to this subject. The markers used for this purpose were various; Somatic Incompatibility (SI), particularly well adapted to this fungus, remained the basic method. Mating-type alleles were used as markers in about ten studies in the eighties; more recently, isozymes were used by Rizzo and Harrington (1993), RAPD by Smith et al. (1992) and Guillaumin et al. (1996), RFLP of mtDNA in several studies after Smith et al. (1990), and RFLP of ITS by Schulze et al. (1997).

These studies aimed at solving several problems in the fields of ecology and epidemiology, particularly the respective roles of vegetative dissemination and sexual reproduction for different *Armillaria* species and in different conditions: *Armillaria* is characterized by active vegetative growth, both through subterranean rhizomorphs and through mycelium within the roots of living plants and stumps. The role of the basidiospores is less clear; however, it is generally admitted that they are at the origin of the new infection foci. They are also the source of genetic diversity.

We must admit that we ignore the exact site where the basidiospores of *Armillaria* spp. germinate in natural conditions. In contrast with *Heterobasidion*, this site is unlikely to be the section of fresh stumps: artificial infection of stumps by basidiospores was completely unsuccessful in a majority of attempts and succeeded at very low rates in some of the experiments of Rishbeth (1964, 1970). The statement that the first infection foci are due to basidiospores rests mainly on indirect arguments: the presence of a few *Armillaria* foci in first-rotation plantations (Rishbeth 1978, 1988), the high number of small genets observed after the clearing of a forest (Hood and Sandberg 1987, Legrand et al. 1996). It is noteworthy that in the case described by Legrand et al. (1996), all the stumps had been removed from the stand 8 years before the mapping of the genets.

If the spatial characteristics of the genets have been the subject of many investigations, it seems that no study has been devoted to the evolution of the genets in course of time. These 'diachronic' studies, which require that the researchers conserve the same research programs over a long period, do not fit in with the present organisation of Research in most countries. In all studies about the genets of *Armillaria*, the evolution of the genets in time has just been inferred from their spatial characteristics (number, size, distribution, overlapping between the genets of the same of different species, etc.).

From 1991 to 1994, we conducted in the mountains of Central France a study which had for main objective the analysis of the biological strategy of *Armillaria ostoyae*. This investigation involved the mapping of the genets of *Armillaria* spp. on four forests with different characteristics and a different history. (Legrand et al. 1996, Guillaumin et al. 1996). It had been decided to prolong this work by repeating the mapping of the genets every four years on three small plots cut up within two of the large parcels which had been originally studied.

MATERIAL AND METHODS

Sites and procedure

Aix-la-Marsalouse: the site is situated in the Département Corrèze (region Limousin), at altitude 760 m. A mixed forest where Scots pine was the dominant species was cut in 1984; the stumps were uprooted with a bulldozer and a natural seedling of Scots pine was left to grow. The seedlings began to die in 1992 as a result of *A. ostoyae* infection. The genets of *A. ostoyae* were mapped after isolation of the fungus from the tap roots of the dead or dying trees. In one half of the stand, isolation was carried out from all dead or dying trees. In the other half, two samples were taken at random in each of the 40 x 40 m squares. As a result of the mapping (Legrand et al. 1996), 38 small, non overlapping genets, were detected on the whole stand. Later, two small plots were defined within the stand, at two places where the genets seemed particularly intricate: the plot 'Aix-ouest' (50 x 30 m) was cut up in that half of the parcel where the sampling had been exhaustive in 1992, and the plot 'Aix-est' (35 x 25 m) in the other half. The *Armillaria* genets were mapped on these two plots in 1996 and 2000, the isolates being obtained again from the roots of the dead and dying pines.

Thus in the plot 'Aix-ouest', the genets of *A. otoyae* were mapped precisely three times, in 1992, 1996 and 2000; in the plot 'Aix-est', the genets were mapped at a large scale in 1992 and more precisely in 1996 and 2000.

Col de Ceyssat: the site is situated at an altitude of about one thousand meters, in the Département 'Puy-de-Dôme' (region Auvergne). It was originally covered by a beech forest, which was cut in 1931 and replaced in 1933 and 1935 by a silver fir plantation. The beech remained as stump sprouts and seedlings. *A. gallica* and *A. cepistipes* are present as saprophytes, *A. ostoyae* either as a saprophyte or as a weak parasite.

An approximate map of the *Armillaria* genets was drawn in 1992 on the whole stand on the basis of a loose sampling of both rhizomorphs and stumps (mycelium and fruitbodies). A small plot of 400 m^2 (four squares

10 x 10 m) was then designed within the stand and was mapped in 1996 and 2000: all the stumps were observed for the presence of *Armillaria*, which was then isolated. In addition, the subterranean rhizomorphs were sampled at 10 points, according to Fig. 2: five rhizomorphs were sampled at random at each of the 10 points. Isolates were obtained from the 50 rhizomorphs and then distributed among the genets.

Thus, on this plot of 'Col de Ceyssat', like at 'Aix-est', the genets were mapped at a large scale in 1992, and precisely in 1996 and 2000.

Armillaria isolation

The isolation was carried out from rhizomorphs, root taps of young pines and fir stumps using the current methods of the laboratory, which involve the culture on a specific medium "MAT" (Malt / Antibiotics / Thiabendazole) (Guillaumin 1977, Legrand et al. 1996).

Distinction of genets

The genets were distinguished with the 'Somatic Incompatibility Method' (SI) used in a number of previous studies. This method proved to be highly discriminant and to give clearcut results (Guillaumin et al. 1991, 1996).

In 2000, the genets detected at Aix-ouest and Aix-est by the SI method were also analysed by RAPD (Random Amplified Polymorphic DNA). DNA extraction and RAPD procedure were conducted as described by Guillaumin et al. (1996). Four decameric primers were used: R25, R28 and UBC 31, designed by Smith et al. (1992) and OPD20 (sequence: ACCCGGTCAC), from the Kit operon Co., selected by Zolciak et al. (1997).

RESULTS

Aix-la-Marsalouse - Plot 'Aix-Ouest'

In 1992, five genets of *A. ostoyae* were present on the plot (C15, C16, C17, C18, C20). Each pine was infected by only one genet , therefore the five genets could be considered as non overlapping.

In 1996, the same five genets were found (Fig. 1A), a sixth, new genet (Cx) was represented by only one isolate (one tree). The borders between the main five genets had not been considerably modified from 1992 to 1996, however C17, represented in 1996 by 23 isolates, had progressed, mainly at the expense of C18.

In 2000, C15, C16, C17, C18, C20 were found again (Fig. 1B), Cx was not found, C16 and C18 were represented by a small number of isolates (each four) and occupied a very small territory; these two genets could be considered as being in process of extinguishing. In addition this territory had moved from 1992 to 2000: in 2000, C18 was located at a place which ,in 1992, was a part of the territory of C17. The borders between the three main genets C15, C17, C20 (represented by 14, 24 and 7 isolates, respectively), were not very different from the situation of 1992.

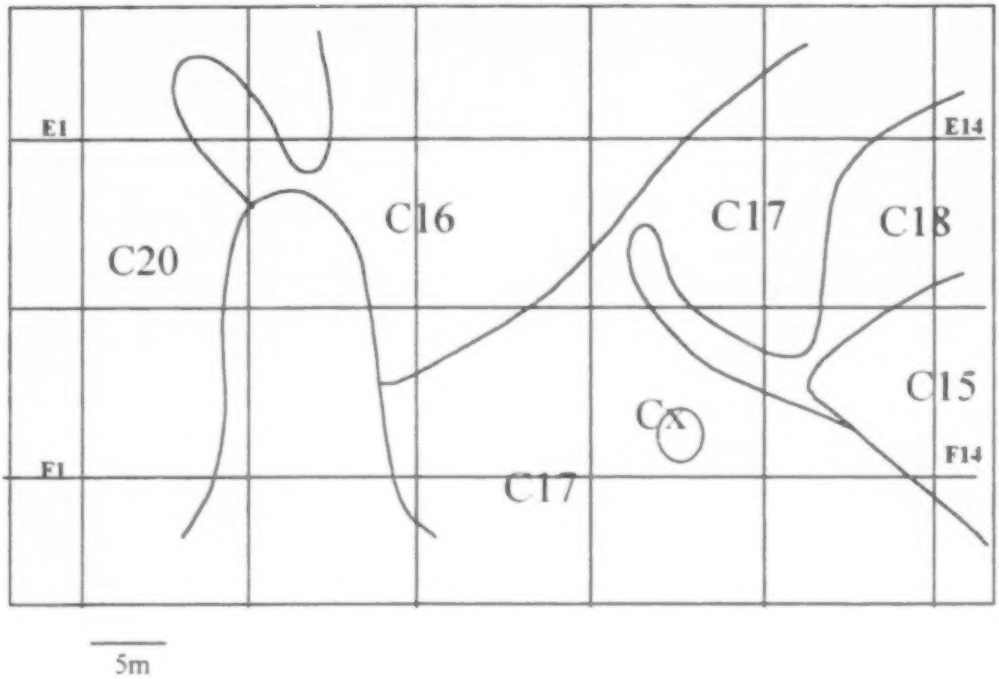
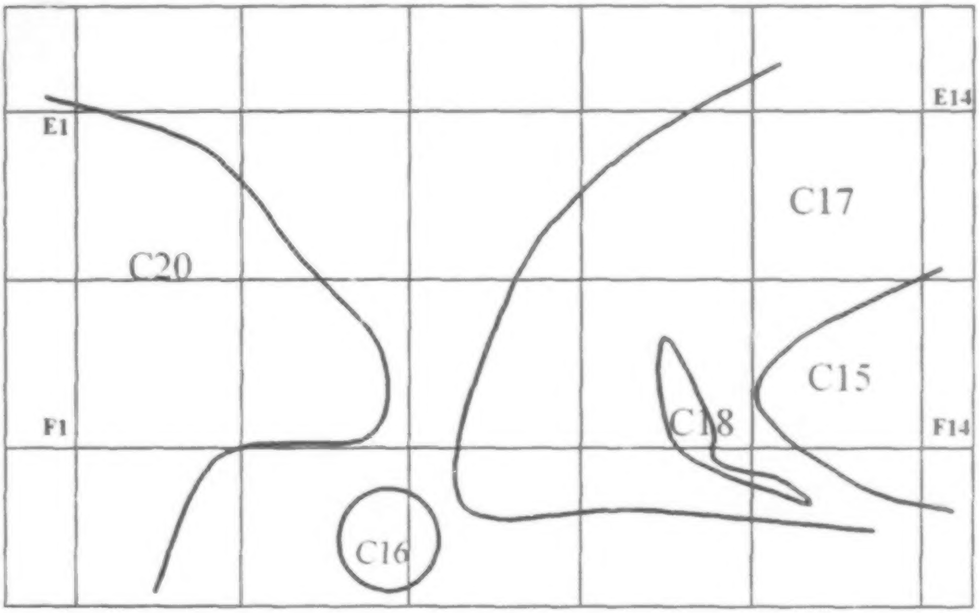

E1
E14
C20
C16
C17
C18
C15
Cx
F1
F14
C17
5m

Figure 1A. Map of the genets of *A. ostoyae* in 1996.


E1
E14
C17
C20
C15
C18
F1
F14
C16

C15, C16, C17, C18, C20 , Cx : genets

E1, F1, E14, F14 : gridmarks

Figure 1B. Map of the genets of *A. ostoyae* in 2000.

Aix-la-Marsalouse - Plot 'Aix-Est'

In 1992, three genets C36, C37, C38, had been detected. The mapping in 1996 detected an additional genet (Cy) and showed that C37 was dominant, with 22 isolates, while C36, C38 and Cy were restricted to small areas, with one, two and four isolates, respectively. The mapping of 2000 revealed the presence of the same four isolates as in 1996. C37 remained the dominant genet, however, C38 and Cy had extended at the expense of C37 (number of isolates in 2000: C37: 13, C36: 1, C38: 7, Cy: 7).

RAPD analysis of the genets of 'Aix-Ouest' and 'Aix-Est' in 2000

The RAPD analysis was carried out in 2000 on one isolate of each of the nine genets found at Aix-Ouest and at Aix-Est (excluding C36 but including Cx which had been conserved in pure culture). The analysis gave almost the same results as the SI method: taking together the bands obtained with four different decameric primers, eight different genotypes could be distinguished, while the SI method distinguished nine genotypes: the genets C15 and C18 of Aix-Ouest, clearly distinguished by the SI method, appeared identical by the RAPD analysis.

Col de Ceyssat

The two species *Armillaria gallica* and *A. ostoyae* were present on the plot. In 1992, five genets had been detected: E1 and E4 (*A. gallica*), C1, C2 and C3 (*A. ostoyae*). In 1996 and 2000, the sampling was carried out from both the mycelium present in the stumps and the rhizomorphs harvested at the 10 gridmark points.

Stumps

In 1996, 65 stumps were present on 400 square meters, among which 25 showed the presence of *Armillaria*. Only 12 isolates were obtained, belonging to the three genets of *A. ostoyae* C1, C2, C3 and the two genets of *A. gallica* E1 and E4 already detected in 1992.

In 2000, 68 stumps were found on the same area, among which:

- 13 recent, non decaying stumps, with their sections generally healed by cambial callus.
- 25 stumps with advanced decay, caused by dry or wet rots different from *Armillaria* rot
- 30 more or less decaying stumps showing the presences of *Armillaria* (mycelial fans, black-lines or heart rot). The isolation carried out from these stumps succeeded for 24 of them, resulting in 11 isolates of the genet C1 (for two of these stumps, C1 had already been isolated in 1996), seven of C2 (already isolated in 1996 from one stump), two of C3, three of E1, one of E4. It failed for six samples, including three stumps for which the isolation had been successful in 1996, giving C1 or C2.

Rhizomorphs

In 1996, the 52 subterranean rhizomorphs harvested at the ten sampling points were belonging to E1 (24 rhizomorphs), E4 (23), C1 (2) and C3 (1). The rhizomorphs of E1 and E4 were found together at 9 points, E1 was lacking at one point and E4 at another point. In 2000, the 49 rhizomorphs harvested at the same places were distributed between E1 (26 rhizomorphs), E4 (20), C1 (2) and C3 (1), E1 was pesent at all ten points, E4 was lacking at a point (not the same as in 1996). The analysis of the detailed results for 1996 and 2000 showed that the number of the rhizomorphs of E1 and E4 observed at each of the ten sampling points was not significantly different from the data given by the binomial distribution.

Size and evolution of the genets of A. ostoyae

The genets C1 and C2 remained small, however, their size increased between 1996 and 2000. These two genets are now coming to contact. C3 remains at the limit of the studied area (Fig. 2).

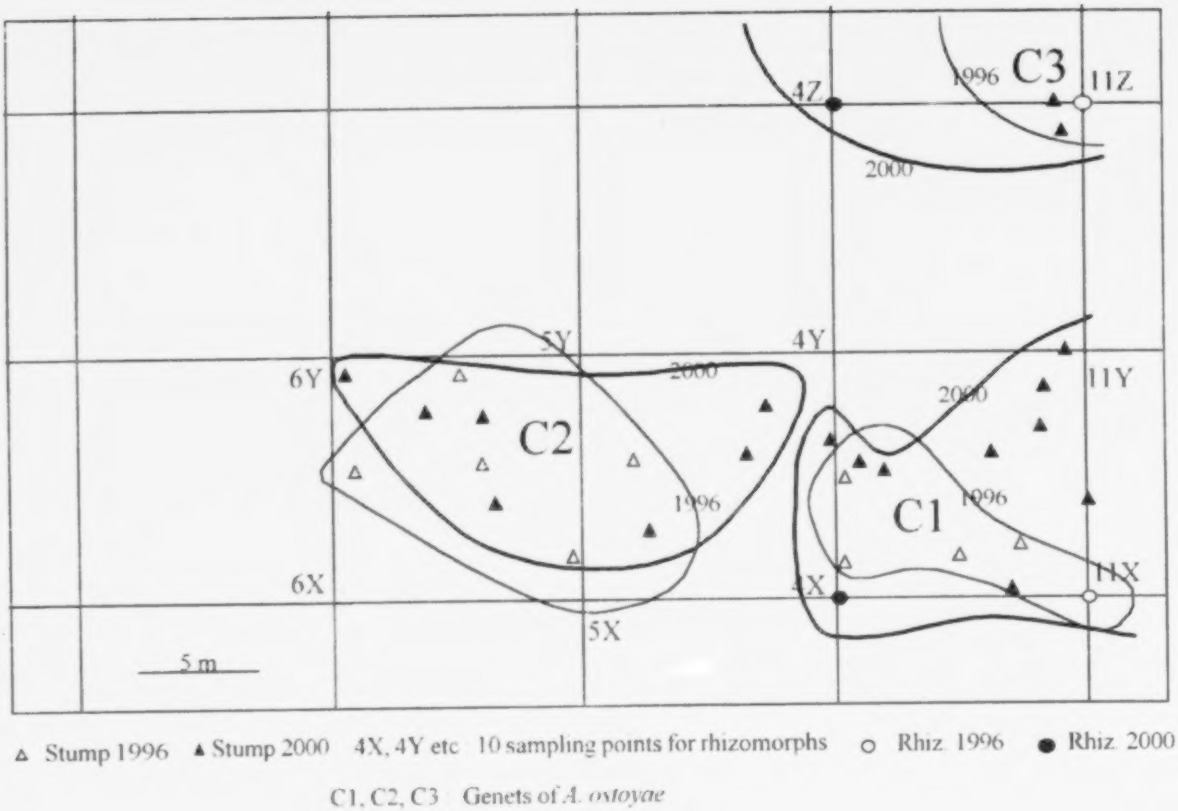

C3
1996
2000
4Z
11Z
C2
C1
4Y
5Y
6Y
11Y
6X
4X
5X
11X
5 m
△ Stump 1996 ▲ Stump 2000 4X, 4Y etc 10 sampling points for rhizomorphs ○ Rhiz. 1996 ● Rhiz. 2000
C1, C2, C3 Genets of A. ostoyae

Figure 2. Map of the genets of *A. ostoyae* at Col de Ceyssat in 1996 and 2000.

DISCUSSION AND CONCLUSION

The first conclusion concerns the pathogenicity of *A. ostoyae* towards Scots pine. In France, *A. ostoyae* is considered as a pathogen of young Scots pines and the susceptibility of this conifer seems to decrease with the age of the trees. Adult trees generally suffer very low damage, except in particular conditions (overaged trees or inadaptation to the site). At Aix-la-Marsalouse, surprisingly, no decrease of pathogenicity was observed over a period of sixteen years. However in 2000, the most vigorous trees generally did not show symptoms of infection by *A. ostoyae*: the dead or dying trees were less than 2.5 m in height.

The second conclusion is in the field of methodology. The study showed that the Somatic Incompatibility method can be used in diachronic studies: the diploid isolates representative of the different genets which had been conserved eight years in the collection (pieces of mycelium on agar stored in water at 4°C in the fridge) showed clear positive or negative reactions when paired with freshly isolated strains; none of these testers had degenerated.

RAPD with four primers appears slightly less discriminant than Somatic Incompatibility, since two genets of Aix-ouest distinguished by SI showed identical bands with all four primers used in RAPD. This number of primers can seem low for such a study, however these primers had been selected in previous studies as generating a high level of polymorphism; in the present study, a total of 23 bands appeared potentially polymorphic among the nine genets analyzed.

The ecological situation of *Armillaria* in the stand of Aix-la-Marsalouse is particularly simple, since: i) only young trees were present, originating from natural germination in 1984 or later, ii) all the stumps from old trees had been uprooted in 1984 when the stand had been cleared, iii) *A. ostoyae* was the only *Armillaria* species present, iv) this species was represented in 1992 by a number of non overlapping genets which each covered a small area and were probably very young (posterior to the clearing in 1984).

The evolution of the genets of *A. ostoyae* at Aix-ouest over eight years appeared very slow: the five genets present in 1992 were still present in 2000 (two of them with very reduced territories) and no additional genet appeared during this period. C16 and C18 are probably in a process of elimination either by natural selection or simply by chance: if the genets are very small and if the fungus is present mainly as mycelium in root systems, the respective location of infected trees and sound trees and stumps susceptible to infection can favour the extension of certain genets and harm others, a process similar to genetical shift. The two genets which are in the way of disappearing have also moved in course of time: these small genets behave like a forest fire which destroys the resource behind while invading new areas.

At Aix-est, where three genets of *A. ostoyae* had been detected, the evolution between 1996 end 2000 was in favour of two small genets, in the prejudice of the larger one.

At Col de Ceyssat, the ecological situation is more complex: this station had been covered by a beech forest until 1935, then a silver fir plantation was established. Partial clearings and storms have given rise to a number of silver fir stumps. Two *Armillaria* species are present on the small plot which was followed up in the course of time: *A. gallica* and *A. ostoyae*. Two genets of *A. gallica* and three genets of *A. ostoyae* were detected in 1992, and these five genets were mapped in 1996 and 2000.

The results obtained confirm, after many other studies, that *A. gallica* and *A. ostoyae* have different biological strategies: *A. gallica* is present mainly as long, thick, robust perennial subterranean rhizomorphs, and is more rarely found as mycelium in fir stumps. This species (and also *A. cepistipes*), is able to colonize both hardwood and conifer stumps from rhizomorphs, however this colonization is rather a rare event taking into account the large quantity of rhizomorphs which cover the surface of the roots. However, *A. gallica* and *A. cepistipes* efficiently exploit their food bases for rhizomorph initiation but these two species can also grow without food bases, by internal reallocation of metabolites (Mohammed 1987). So, they are adapted to active exploration of the soil in search for rare food bases. In the present study, five rhizomorphs were harvested at each sampling point. This procedure permitted to study the possibility of overlapping of the genets, which in many previous studies has been frequently hidden by the sampling of only one rhizomorph at each sampling point. The two genets of *A. gallica*, E1 and E4, were present as rhizomorphs over the whole plot, both genets were present on a majority of sampling points, the relative number of rhizomorphs of E1 or E4 observed at all the sampling points was not significantly different from a random distribution. So, the overlapping of the two genets was complete: it appears that the rhizomorphs of two different genets can intercross without reciprocal inhibition. When an *Armillaria* species exists mainly in the form of rhizomorphs and has been present for a long time in a site, each of the different genets of the species tends to invade the totality of the available surface, with as a consequence a complete overlapping between two, or more, genets. This fact is probably true also for the genets of two different species with similar biological strategies, for instance *A. gallica* and *A. cepistipes* (Legrand et al. 1996).

These large genets of *A. gallica,* and of *A. cepistipes* which is also present in other parts of this studied stand, are probably a 'legacy' of the beech forest, which occupied the site of 'Col de Ceyssat' before 1931. These species have not been eliminated by the evolution of the site to a fir forest after 1935, because i) the rhizomorph network can survive through internal transfers of metabolites, ii) these species can also colonize conifer stumps, although less readily than hardwood stumps, iii) the plantation of firs was made without removing the beech stumps, iv) beech sprouts continue to grow as an understorey in this stand.

By contrast, in the same site 'Col de Ceyssat', *A. ostoyae* is frequent as mycelium in fir stumps, but rare in the form of rhizomorphs in soil. The rhizomorphs of this species are known to be shorter and to grow more slowly than those of *A. gallica*, and they are also known to exploit less efficiently their food bases. The colonization of the conifer stumps by this species probably proceeds in many cases from latent infections on living roots of conifers (Delatour and Guillaumin 1995); after the tree dies or is cut, *A. ostoyae* rapidly invades the total root system from the latent infections, having a considerable advantage on a species such as *A. gallica* which has to colonize the stumps from its external rhizomorphs.

The three genets of *A. ostoyae* present on the plot were small and their area increased between 1996 and 2000. These genets, represented mainly by mycelium in the fir stumps, did not overlap, the territories of C1 and C2 were just coming to contact in 2000. It is likely that these small genets are young, they probably settled recently, after the plantation of firs.

The general impression given by the study is a great stability of the genotypes: in the three plots investigated, a total of 14 genets of *Armillaria* spp. had been detected in 1992. Only two additional genets were detected in 1996; one of them (Cx at Aix-ouest) was present in only one root system, the other (Cy at Aix-est) had probably remained undetected because the sampling of 1992 was not extensive in this half of the parcel. In 2000, the same genets as in 1996 were found, with the exception of Cx which had disappeared.

The appearance of a new *Armillaria* genet seems to be a very rare event, particularly if one considers the high number of basidiospores which land on these forests in autumn. It seems that the formation and survival of a diploid in nature after the mating of two compatible haploids requires very precise conditions. The exact site of this appearance is unlikely to be the horizontal section of the stumps after cutting the trees; in the present study, none of the fresh stumps formed between 1996 and 2000 at 'Col de Ceyssat' were, in 2000, infected by new genets; these new stumps with *Armillaria* were colonised by previously established genets of *A. ostoyae*, possibly from latent infections made when the trees were alive. It is likely that the conditions necessary for appearance of new genotypes are better fulfilled when the forest ecosystem is disturbed, particularly when a forest is clearcut and replaced by seedling: the great number and low area of the genets of *A. ostoyae* present in 1992 at Aix-la-Marsalouse was consistent with the hypothesis that a majority of these genets had appeared just after the events of 1984.

ACKNOWLEDGEMENTS

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POPULATION STRUCTURE AND MATING SYSTEM OF *CLIMACOCYSTIS BOREALIS*

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SUMMARY

Climacocystis borealis is frequently associated with butt rot of *Picea abies* in Switzerland. It is essential to understand the biology of this fungus to develop adequate control strategies. Thirty-seven basidiocarps were collected from the standing bases of snapped trees and stumps of Norway spruce in the region of Zurich. Mono-basidiospore isolates and isolates from the trama were paired with each other to study the mating system. Three to five weeks of incubation at 22°C were needed to get distinct reactions in vegetative compatibility tests. The basidiocarps belonged to 13 vegetative compatibility groups (VCGs). Basidiocarps of up to three VCGs were present on one stump indicating multiple infections. Fruit bodies from different sites and stumps belonged to different VCGs except in one instance. Basidiocarps from two stumps standing in a distance of 10 m from each other belonged to the same VCG, possibly due to an infection by root grafts. The macroscopic differentiation of the various types of interactions between the mycelia of monospore isolates was difficult. Whereas compatible interactions could clearly be differentiated from incompatible ones by the formation of clamps, differentiation between hemicompatible and incompatible reactions was a problem. The proportion of compatible: presumed hemicompatible: presumed incompatible monospore-isolate pairings was 5:19:17 and 5:19:15 for two of the basidiocarps. These proportions deviate from 1:2:1, the ratio expected for a fungus with a tetrapolar mating system.

Keywords: mating system, vegetative compatibility

INTRODUCTION

C. borealis is frequently associated with butt rot of *Picea abies* in Switzerland (Graber 1996) but largely neglected by contemporary research. We need to understand its biology to develop adequate control strategies. Especially, it is essential to discover the basic mechanisms of reproduction and spread of *C. borealis*. The aim of this study was to describe the spatial distribution of heterokaryotic mycelia within and between different substrates and to reexamine the finding of Robak (1932) who described the mating system of *C. borealis* to be tetrapolar.

MATERIALS AND METHODS

Thirty-seven basidiocarps were collected at seven sites within an area of 140 km^2 in the region of Zürich from 12 substrate units, i.e. standing bases of snapped trees or stumps of Norway spruce (Fig. 1). Isolates from the trama of each basidiocarp were prepared within 24 hours after collection on 2% (w/v) malt agar (20 gl^{-1} malt extract, 15 gl^{-1} agar) (MEA) amended with 230 mgl^{-1} thiabendazol (2-[1,3 thiazol-4-yl]-benzimidazol) dissolved in 1 ml lactic acid (TMEA). Mono-basidiospore isolates were received from two genetically different basidiocarps. The fresh basidiocarps were fixed 10 cm above the surface of TMEA and allowed to release spores for 2, 10 and 20 s. Germinating spores were transferred to MEA one day later. Each monospore isolate was checked for the absence of clamp connections to confirm the origin from one single basidiospore. Cytology of the basidiospores was examined by DAPI staining (Campbell and Duffus 1988).

Trama isolates from basidiocarps originating from the same substrate unit were paired first. Then, one representative isolate of each vegetative compatibility group (VCG) from each substrate unit was paired with each representative from the other substrates. Monospore isolates were paired with each other in all possible combinations. Pairing experiments were performed on MEA. The inoculi were placed in a distance of 10 mm from each other and consisted of colonized 4-mm-diam plugs of MEA removed from the margin of an actively growing colony. Incubation occurred at 22°C in the dark.

RESULTS

The evaluation of the VC tests was possible only after an incubation period of at least five weeks. The 37 basidiocarps belonged to 13 VCGs. Fruitbodies of up to three VCGs were present on a single stump indicating multiple infections (Fig. 2). Only one genet was detected on seven of 12 stumps or bases of snapped trees. Basidiocarps from two stumps standing close to each other belonged to the same VCG, possibly due to an infection by root grafts.

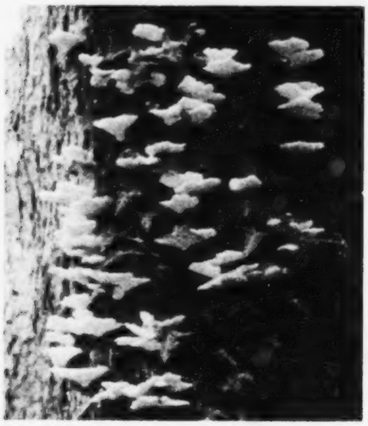


Figure 1. Basidiocarps of *C. borealis* on the base of snapped tree.

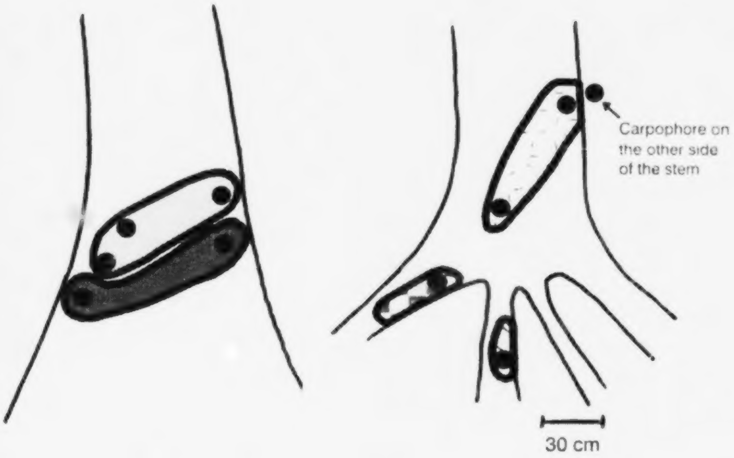
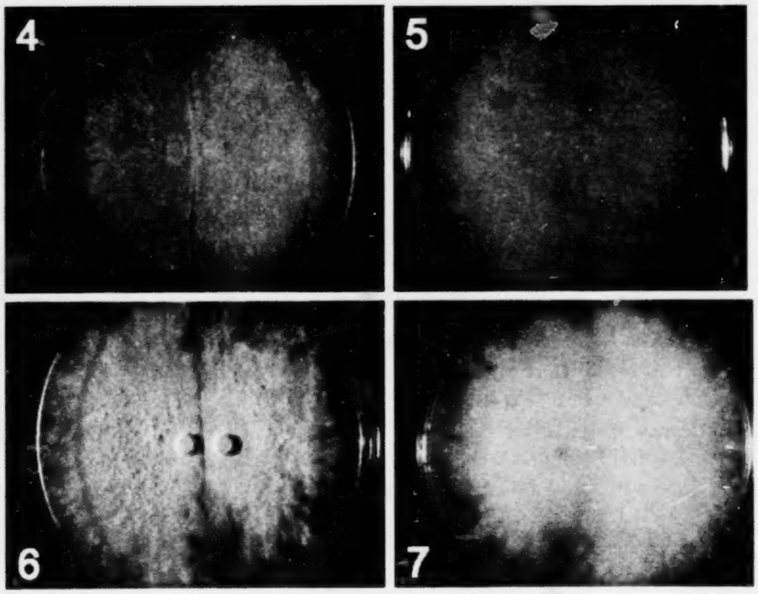


Figure 2. Sketches of two stem bases of Norway spruce showing the positions of the basidiocarps (•) and the approximate areas colonized by each VCG (differently shaded areas).

The basidiospores of *C. borealis* were constantly uninucleate and germinated readily (Fig. 3). They did not survive freezing in water at -18°C.

Compatible interactions between monospore isolates could clearly be differentiated from hemi- or incompatible ones by the presence of clamps. The differentiation between hemi- and incompatible interactions was, however, difficult (Figs 4-7). Hemicompatible interactions were often characterized by incomplete clamps in the interaction zone. Incompatibility was assumed when a dense white demarcation line was visible from the reverse of the Petri dish (Fig. 5). The proportion of compatible: presumed hemicompatible: presumed incompatible monospore-isolate pairings was 5:19:18 and 5:19:15 for the two basidiocarps (Table 1).

Figure 3. Basidiospores of *C. borealis* stained with DAPI. The big spot in each spore is the nucleus, the small spots indicate mitochondria. Bar = 10 µm.



Figures 4-7. Interactions between monospore isolates on 2% MEA. 4. Surface view of a presumed incompatible interaction. 5. View of the same interaction as in 2 from the reverse of the Petri dish. 6. Surface view of a presumed hemicompatible interaction. 7. View of the same interaction as in 4 from the reverse of the Petri dish.

DISCUSSION

Compatible and incompatible interactions between heterokaryons and compatible interactions between monospore isolates were easy to recognize on 2% (w/v) malt extract agar. A more sophisticated method is, however, needed to allow differentiation between hemicompatible and incompatible homokaryon pairings.

The proportion of compatible interactions was rather low for a fungus with a tetrapolar mating system. In addition, some of the results were contradictory. Either the experimental conditions were not optimal or *C. borealis* does not have a tetrapolar mating system. Several basidiomycetes without a bi- or tetrapolar mating system are known (Rayner and Turton 1982, Rogers et al. 1999).

Table 1. Pairings of monospore isolates originating from two basidocarps. Upper triangular matrix = basidocarp no. 1; lower triangular matrix = basidiocarp no. 2. Interactions: + = compatible, - = presumed incompatible, h = presumed hemicompatible, ? = hemi- or incompatible.

Monospore isolate number	1	2	3	4	5	6	7	8	9	10
1	▨	h	h	-	-	h	■	?	h	h
2	■	▨	?	h	-	-	-	h	h	h
3	■	h	▨	?	-	?	-	h	h	h
4	h	h	■	▨	■	■	-	h	h	h
5	h	h	-	h	▨	■	-	?	-	-
6	h	h	-	h	■	▨	-	h	-	-
7	h	-	-	h	-	-	▨	■	?	-
8	h	h	?	?	h	-	-	▨	h	h
9	h	-	h	-	-	-	h	-	▨	h
10	h	?	-	-	h	-	-	■	-	▨

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THE MATING BEHAVIOR OF *STEREUM SANGUINOLENTUM*

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EXPANDED SUMMARY

The conception of *S. sanguinolentum* to be either apomictic (= parthenogenetic) or homothallic (Robak 1942) is hardly coherent with the high number of sympatric and allopatric vegetative compatibility groups (VCGs) (Holdenrieder and Stenlid, unpublished). In this study, we tried to find clues whether meiosis is really the exception in *S. sanguinolentum* as suggested by Robak (1942).

Basidiocarps of *S. sanguinolentum* were collected from conifer logs in the region of Zurich, Switzerland. Monospore and trama isolates were prepared and paired. The hymenial surface was studied by scanning electron microscopy. Thin-sections of hymenia were stained with DAPI and studied under epifluorescence to examine the behavior of the nuclei in the basidia.

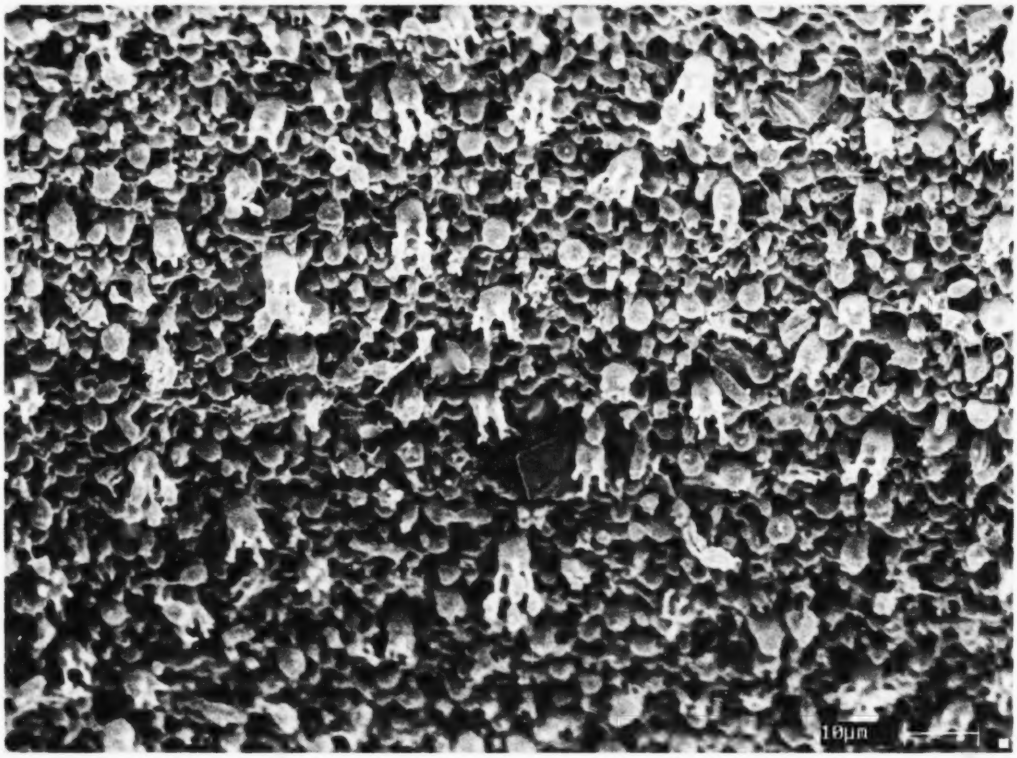
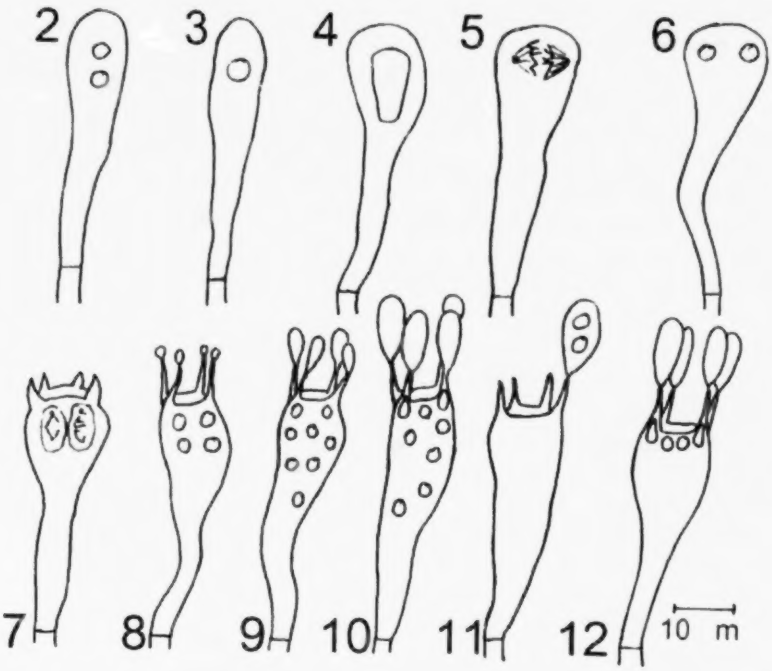


Figure 1. Scanning electron micrograph of the central part of a mature hymenium of *Stereum sanguinolentum* with a high number of regularly four-spored basidia.

Intra-fruit body monospore pairings were always compatible. In contrast, inter-fruit body pairings were incompatible except in one instance independently of whether monospore or trama isolates were paired. The basidia were regularly four-spored (Fig. 1). Most of the basidiospores were binucleate. Some possessed, however, one, three or four nuclei. Ten percent of the spores appeared to be empty. Karyogamy, meiosis and postmeiotic mitosis were regularly observed in the basidia (Fig. 2-12). The resulting eight nuclei were usually located in the basidia before they moved through the sterigmata into the spores. Sometimes, nuclei were observed in the sterigmata without having undergone a postmeiotic mitosis. Based on these observations *S. sanguinolentum* is regarded to be an amphithallic basidiomycete.



Figures 2-12. Meiotic stages observed in the basidia. 2. Dikaryon (prekaryogamy); 3. Zygote; 4. Prophase I; 5. Meta-, anaphase I; 6. Telo-, interphase I; 7. Anaphase II; 8. Telophase II; 9. Basidium with eight nuclei after postmeiotic mitosis; 10. Migration of nuclei through the sterigmata. 11. All nuclei in the spores, basidia empty; 12. Migration of nuclei through the sterigmata prior to the postmeiotic mitosis.

Whereas Robak (1942) detected meiosis only rarely, it was regularly observed in this study. Thus, *S. sanguinolentum* is either a homothallic or amphithallic species. However, the high diversity of sympatric VCGs makes homothallism improbable. It may, thus, be best to regard *S. sanguinolentum* as amphithallic (Petersen 1995). Recombination could occur either by mating between homokaryons originating from monokaryotic basidiospores or by parasexual processes (Anderson and Kohn 1998).

Keywords: meiosis, vegetative compatibility, amphithallism

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DEVELOPMENT OF SIMPLE SEQUENCE REPEAT (SSR) MARKERS IN *ARMILLARIA OSTOYAE*

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SUMMARY

A modified hybridisation strategy was used to construct a microsatellite enriched library from DNA of *A. ostoyae*, a serious root pathogen on pine. Sequence characterisation of 19 random clones revealed 12 distinct loci harbouring a repetitive motif. Primer design from the flanking regions allowed for their development as PCR based markers. Polymorphic assessment at both the population and global levels revealed levels of variation useful for genetic studies. The level of cross-species amplification observed with closely related *Armillaria* species was high, raising the possible exploitation of these primers across the genus.

Keywords: *Armillaria*, microsatellites, PCR markers, phytopathogenic fungi

INTRODUCTION

A. ostoyae (Romagn.) Henrik, a serious root pathogen of conifer species, is particularly prevalent on maritime pine (*Pinus pinaster* Ait.) in south-west France where its impact has important ecological and economical implications. Sub-terranean vegetative hyphal growth is considered the predominant means of disease spread, whereas the significance of sexual reproduction to the epidemiology of the disease remains unclear. In the absence of fully effective conventional or biological control measures, evaluation of the extent to which sexual and asexual reproduction affects population structure and disease dissemination through molecular marker analysis may help develop new pathogen management strategies. Since microsatellite (Simple Sequence Repeats-SSRs) are co-dominant, multi-allelic markers which typically exhibit high levels of variability, they are continuing to find increasing applications in fungal genetic diversity and population studies (Moon et al. 1999; Neu, et al. 1999; Tenzer et al. 1999; Burgess et al. 2001). Because of their polymorphic versatility, we have initiated a programme to isolate and characterise SSRs in *A. ostoyae,* for the development of PCR based SSR markers, to assist studies of genetic diversity and population structures and dynamics in this species.

MATERIALS AND METHODS

Enrichment strategy

Rhizomorphs of strain CA3.99, isolated from carpophore material collected from a dead maritime pine from La Caguillouse district (Pontenx-les-Forges, Les Landes), were axenically cultured on pieces of organicaly grown orange fruits over sterile tap water (Jacques-Felix 1968). DNA was prepared from washed lyophilised material using a kit (Puregene®, Gentra Systems). A microsatellite enriched library was then constructed following the procedure of Edwards et al. (1996), as modified according to Butcher et al. (2000). This included an additional round of hybridisation enrichment using di (GA and CA) and tri (GAC, GAA, GCC, CAA, GCT and ACC) oligonucleotide motifs. Only the di-nucleotide motifs yielded PCR product following second round hybridisation. Enriched PCR product was purified (Microspin™ S-400 HR column, Amersham Pharmacia) prior to ligation into pGEM-T (Promega) and transformed into *Escherichia coli* DH5α. Inserts of 89 randomly pre-selected individual clones were amplified with vector specific primers and screened for the presence of SSRs by chemiluminescent dot hybridisation (Boehringer). Strong signals were obtained for 32 (of which 27 had inserts ≥

600 bp) using the CA motif, representing an overall enrichment efficiency of 36 %. No signal was obtained using the GA repeat. Seventeen out of 19 selected clones were shown to harbour a SSR following cycle sequencing (Applied Biosystems). Five redundant clones were identified following multiple sequence alignments, with two harbouring degenerate CA rich sequences. Forward and reverse primers were designed from SSR flanking regions of the remaining 12 sequences using the computer PRIMER software (version 0.5, Whitehead Institute).

PCR reactions (25 µl volumes) on 5 ng genomic DNA comprised 0.1 mM each primer, 100 mM dNTPs, 1.25 units Taq DNA polymerase (Life Technologies), 200 mM Tris-HCL (pH 8.4), 500 mM KCL, and 1.5 mM $MgCl_2$. Amplification conditions, using a Perkin-Elmer 9700 thermal cycler comprised initial denaturation (3 min at 94°C) followed by 30 cycles of 30 sec at 94°C, 30 sec at 58-63°C (empirically defined for each primer pair), 30 sec at 72°C, with a final post extension of 10 min. at 72°C. Success of individual PCR reactions were confirmed by 2% agarose electrophoresis. Each primer pair successfully amplified template originally used to construct the library. Predicted size fragments were confirmed by 6% urea-polyacrylamide gel electrophoresis followed by silver nitrate staining (Sambrook et al. 1989).

Polymorphic assessment

Eighteen *A. ostoyae* isolates representing six Somatic Incompatibility (SI) groups from a 4 ha experimental forest site at Pontenx, Les Landes, were used to assess the polymorphic information content of each marker at the population level. SI testing was performed according to the method described by Korhonen (1978). At a wider level, five isolates of diverse global origin (France, UK, USA and Japan) were assessed. PCR and polyacrylamide gel conditions were as described above. Calculation of allelic diversity was used as a measure of variation. All SSR markers were assessed for cross-species PCR amplification under stringent conditions (65°C annealing temperature) against representative members (two isolates per species) of the *mellea* group; *A. borealis*, *A. cepistipes*, *A. gallica*, *A. mellea*, including *A. tabescens*. All species identities (including all *A. ostoyae* isolates) were confirmed according to the IGS PCR-RFLP method described by Harrington and Wingfield (1995).

RESULTS

Results are summarised in Table 1. Although four markers gave no polymorphism at the population level, higher levels of allelic diversity were observed with globally diverse strains (except for the most polymorphic markers *Ao*SSR21 and *Ao*SSR74, although this may be due more to sample size descrepencies). One to two global isolates failed to amplify a band for five markers and were recorded as having a 'null' allele. SSR profiles were identical within each SI group studied, supporting their consideration as clonal. However, although inter SI group variability provides indirect support that recombination is possible at the population level, only a maximum of five out of six SI groups were differentiated by *Ao*SSR21 and *Ao*SSR74, supporting the view that SI in *A. ostoyae* is polygenic and complex. No clear correlation between motif length and allelic diversity at either level was observed. Similar sized products, of varying levels of amplification intensity (compared with *A. ostoyae*) were observed across all species tested (Table 2), indicating a high degree of inter-specific flanking sequence conservation.

CONCLUSION

PCR based SSR markers have been successfully developed for population and diversity studies in *A. ostoyae*. Frequency of microsatellite enrichment and success in revealing polymorphisms at the population level is comparable to other fungal species where similar strategies have been employed (e.g. Owen et al. 1998; Neu et al. 1999). The almost exclusive CA motif content of clones revealed by our random sequencing approach may reflect a high CA content within the *Armillaria* genome, particularly as all other motifs failed to enrich despite repeated attempts. As both SI and SSR markers appear to assess genetic variation independently, SSR analysis (using markers *Ao*SSR21, *Ao*SSR62, *Ao*SSR74 and *Ao*SSR84) will be used to compliment SI testing of genetic structures of *A. ostoyae* populations within maritime pine plantations from south-west France. Finally, the level of cross-species amplification observed suggests a degree of conservation of inter-specific genome organisation within the *mellea* group, highlighting a number of phylogenetic implications and transfer of *A. ostoyae* defined SSR markers to diversity studies in these taxa (e.g. *A. gallica*, unpublished results of this laboratory).

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We are grateful to Dr J.-J. Guillaumin, INRA, Clermont-Ferrand, for additional global isolates of *A. ostoyae* and *Armillaria* spp., and Dr V. Decroocq, INRA, Bordeaux, for helpful discussions.

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Table 1. PCR primer sequences and characteristics of *Armillaria ostoyae* microsatellite loci. Numbers of alleles and allelic diversity values are calculated separately for a population sample of 18 individuals representing 6 SI groups and 5 global isolates (representing 4 countries (France, UK, USA and Japan) from 3 continents), respectively.

Locus	SSR Motif	Primer sequences (5'-3')	Expected size (bp)	Observed range (bp)[a]	No. of alleles			Allelic diversity[b]		EMBL No.
					Pop.	Global	Total	Pop.	Global	
*Ao*SSR21	$(CA)_{11}$	F:AGCCAGGATGATGTTGATGT R:CCACGAGTGCTTCTACCATA	154	149-163	5	7	12	0.81	0.80	AJ307587
*Ao*SSR22	$(GT)_5$	F:AGATACCATACGAGCGCGAT R:GGCTAGAAATGCAACAGCG	174	172-176	2	3	3	0.00	0.48	AJ307588
*Ao*SSR24	$(GT)_{11}$	F:CACCACGAGTGCTTCTATCA R:TGCTCGGTAATAGGCAGAG	135	132-140	1	4	5	0.00	0.72	AJ307589
*Ao*SSR27	$(CA)_{10}$	F:GCTGGTAATGGGCACTCAT R:GTCAGGGCACAAGCAGAAT	152	150-166	4	5	9	0.67	0.80	AJ307590
*Ao*SSR29	$(CA)_4$•$(CA)_{12}$	F:ACACCCTTGCGTTGTTTATC R:GGTTATAGTAATCGTCCACTGC	128	126-132	2	3	4	0.44	0.64	AJ307591
*Ao*SSR30	$(TG)_8$	F:TCCTTGTTTCTTTCTGCACT R:GGCTGGTAATGGGCACTC	125	121-133	2	5	7	0.00	0.32	AJ307592
*Ao*SSR62	$(CA)_{11}$	F:AGGATGATGTTGATGTGCTT R:CCACGAGTGCTTCTACCATA	150	140-158	4	6	10	0.67	0.72	AJ307593
*Ao*SSR65	$(CA)_7$	F:TAAACCACTACTCAATCCCACT R:GATACTGTCAGGGTGCAAGT	100	94-106	2	5	7	0.00	0.72	AJ307594
*Ao*SSR74	$(GT)_{10}$	F:GCTCACCCTCAAACTTAACA R:GCAGGGCACAAATGAAACTA	103	96-114	5	5	10	0.81	0.72	AJ307595
*Ao*SSR75	$(GT)_{12}$	F:GTAATCGTCCACTGCTCGG R:CATCAACTCAAAACCCTTGC	131	124-136	2	3	4	0.44	0.64	AJ307596
*Ao*SSR76	$(GT)_{11}$	F:ATCAACAGGCGTTCAGATAA R:GCCAACGAACACATAAGC	112	110-116	2	2	4	0.03	0.48	AJ307597
*Ao*SSR84	$(GT)_{11}$	F:ACACCACGAGTGCTTCTACTA R:GCTTGGTAATGGGCAGAG	137	128-150	5	6	11	0.50	0.80	AJ307598

[a] Approximate range of allele sizes amplified across all isolates studied.

[b] Allelic diversity (Nei, 1973) was calculated for each locus by including 'nulls' as separate alleles.

• symbolises unspecified length of sequence.

Table 2. Assessment of all SSR markers for cross-species PCR amplification (at 65° C annealing) against representative species of the *mellea* group, including *A. tabescens*. PCR amplification intensity (as compared with *A. ostoyae*), following 2% agarose gel electrophoresis and Ethidium bromide staining, used as an arbitrary indication of the level of inter-specific flanking sequence conservation. Species are listed according to the ITS PCR-RFLP phylogeney from Chillali *et al.* (1998).

Species	SSR locus											
	*Ao*SSR21	*Ao*SSR22	*Ao*SSR24	*Ao*SSR27	*Ao*SSR29	*Ao*SSR30	*Ao*SSR62	*Ao*SSR65	*Ao*SSR74	*Ao*SSR75	*Ao*SSR76	*Ao*SSR84
A. ostoyae	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. borealis	+	+++	+	+++	-	+++	+	+++	+++	+	+++	+
A. cepistipes	+	+	+	+	+	+	++	+	+++	+	++	+
A. gallica	++	++	+	++	++	++	++	+++	++	+	+	+
A. mellea	+	-	+	+	-	+	+	+	+	+	+	+
A. tabescens	+	-	+	-	-	-	+	++	-	-	-	-

Where the following symbols signify; +++, strong; ++, medium; +, weak; -, no amplification.

SEQUENCE POLYMORPHISM IN LACCASE GENES OF S, P & F TYPES OF *HETEROBASIDION ANNOSUM*

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SUMMARY

Laccases are multicopper enzymes, which have been implicated to play a role in lignin breakdown and wood degradation. Earlier comparative studies revealed that P-types of *H. annosum* produced significantly more laccases than S-type, which also correlated with their higher wood degrading ability. Therefore, how the nucleotide sequence of the gene encoding laccase in all three intersterility groups (IGs) of *H. annosum* (S, P & F-types) differed from each other was investigated. Using oligonucleotide primer, the gene encoding laccases was isolated by PCR from genomic DNA of 28 isolates of S, P & F types of *H. annosum* that originated from different geographic regions (Europe, Asia and North America) together with two Australian isolates (*Heterobasidion araucariae* and *H. insulare*). Sequence alignment of the laccase genes shows polymorphisms within all IGs. More sequence polymorphism appeared within European F and S-isolates. Within the group, there were no significant differences between the S and F-isolates of European strains. Sequence alignment also highlighted differences in a few nucleotide bases and amino acids among the isolates. Phylogenetic trees showed that some European S-isolates are grouped together closely with a few North American S-isolates. The North American and European P-isolates were grouped together. The S and F-types appear to be genetically closer than to P-types.

INTRODUCTION

Heterobasidion annosum is recognized as a major pathogenic necrotrophic fungus of both young seedlings and mature trees, where it produces characteristic root and butt rot (Hodges 1969; Asiegbu et al. 1998). Economic losses attributable to *H. annosum* infection are estimated to exceed 790 million ECU *per annum* in Europe (Woodward et al. 1998). *Heterobasidion annosum* has been separated into different subtypes or intersterility groups that are classified as P, S and F, based on the host preference of each type (Scots pine, Norway spruce and Silver fir, respectively). To degrade wood, *H. annosum* uses a complex set of enzymes. Among these enzymes, laccase appears to be the predominant ligninolytic enzyme (Asiegbu et al. 1998). Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper enzymes which catalyze the oxidation of phenolic compounds and are found in both plants and fungi. Laccases are very important in lignin degradation because they are capable of oxidizing and depolymerizing different lignin preparations (Bourbonnais and Paice 1990). Earlier results on wood decay tests revealed that P strains, which possess significantly greater wood degrading abilities than S-isolates, also secreted 5 to 6 times more lignin/cell wall degrading enzyme (Laccase) than the S-isolates under liquid culture conditions (Daniel et al. 1998). We therefore presumed that the differential secretion of laccase enzyme by *H. annosum* strains correlates to their wood decay capability in trees that have been killed. Moreover, how the nucleotide sequences data of the gene coding for the laccase of the three IGs of *H. annosum* strains differed from each other is of considerable interest. In the present work, we have examined sequence polymorphism in laccase genes of selected IGs of *Heterobasidion annosum* strains. By comparing the deduced nucleotide sequence of *H. annosum* laccase and a number of other fungal laccases, inference may be drawn on the functional relevance of the gene in relation to evolutionary relatedness of the isolates.

MATERIALS AND METHODS

Cultures

Fungal strains. In total, 28 isolates of the intersterility group (S, P and F types) of *Heterobasidion annosum* (Fr.) Bref, together with two Australian isolates (*Heterobasidion insulare* and *Heterobasidion araucariae*) were used in this experiment. They were provided by Dr Kari Korhonen, Vantaa, Finland, and Dr. Jan Stenlid, Uppsala, Sweden, who had isolated them and determined them to intersterility group (IG) by somatic incompatibility tests (Korhonen 1978). Dr P.K. Buchanan (New Zealand), provided isolates of *H. araucariae* and *H. insulare* (Murrill) Ryvarden. Two isolates of the F-intersterility group of *Heterobasidion annosum* (Capretti et al. 1990) were provided by Dr Kari Korhonen. The origins of the *Heterobasidion* S, P & F-isolates are presented in Table 1. The cultures were maintained on Hagem agar (Stenlid 1985).

Table 1. S, P and F isolates of Heterobasidion annosum, Heterobasidion insulare and Heterobasidion araucariae were used.

Strain code	Strain Name	Country of Origin	Strain code	Strain Name	Country of Origin
S1	87-179 C-2	Norway	P1	TC 32-1	North America
S2	FSE-3	N. Europe	P2	MJT 1556(B-4)	North America
S3	95125	Russia	P3	FP5	Finland
S4	87 184 C-3	Norway	P4	VG 81	Finland
S5	OH2 8C-6	Switzerland	P5	TC III-3	North America
S6	BR 518 C-2	Sweden	P6	TC III-4	North America
S7	B1081	Japan	P7	Sa 16-4	Sweden
S8	B1314	China	P8	MJT 272	Sweden
S9	TC 122-11	North America	F1	Faf 6-2	Southern Europe
S10	95156	Russia	F2	Faf 5-2	Southern Europe
S11	BC 3-3	North America	F3	Faf 10-2	Southern Europe
S12	Fr 154	Sweden	F4	Faf 7-1	Southern Europe
S13	ORE 103	North America	F5	Faf 4-6	Southern Europe
S14	Sa 192	Sweden	Hi	*Heterobasidion insulare*	New Zealand
S15	95151	Russia	Ha	*Heterobasidion araucariae*	New Zealand

DNA Extraction

Genomic DNA was extracted by using modified methods of Zolan and Pukkila (1986).

PCR Reaction

For PCR, 100 ng of genomic DNA was mixed with gene specific primers (Asiegbu 1998, 2001): (Lac1 5′; AGCAYTGGCAYGGCTTYTTCCA; Lac1 3′; GTCRATGTGGCARTGGAGGAACCA) under the following standard PCR conditions [5 min at 95°C (1 cycle), 1 minute at 95°C, 1 min at 55°C, 2 min at 72°C (30 cycles), and 5 min at 72°C (1 cycle)].

Sequence Analysis

Sequences were aligned with Sequence Navigator. Ambiguously aligned 3′ and 5′ data set were excluded from the initial alignment of 700 bp. Uninformative characters were ignored, and gaps were coded as a newstate. Bootstrapping (1000) replicates and decay indices (Autodecay 2.9.4 with PAUP) were used to determine confidence in the branches.

RESULTS

Aligned sequences of S, P & F isolates of H. annosum, H. insulare and H. araucariae strains were calculated for percentage identity and distance matrix (Table 2). Nucleotide sequence analysis showed that all S-isolates were more closely related to each other. Nordic S-isolates are closely related than Asian and North American strains. Asian strains are very close to each other to North American isolates.

Table 2. Distance and percentage of identity comparing the nucleotide sequence of the laccase gene region of isolates of S, P & F-isolates of *H. annosum, H. insulare* and *H. araucariae.* The top right triangle shows the percentage of identity computed from the sequence alignment in Fig. 1a using the program Sequence Navigator. The bottom left shows distance matrix using the program PAUP Phylogenetic Analysis using parsimony.

Strain code	S1	S3	S5	S6	S7	S8	S9	P1	P3	P8	F1	F2	F3	Hi	Ha
S1	——	99,78	99,54	99,34	97,63	97,26	96,95	91,04	90,62	87,55	97,62	97,22	97,77	86,24	84,75
S3	0,03207	——	98,57	99,14	97,39	96,63	96,30	89,81	90,33	87,55	97,96	96,79	98,03	87,39	84,01
S5	0,02954	0,03881	——	97,71	96,23	95,86	95,69	89.51	90,75	87,22	96,26	96,03	97,71	86,25	86,15
S6	0,00639	0,01292	0,03000	——	96,45	96,46	95,83	89,39	90,85	87,87	96,68	96,23	97,45	85,12	82,92
S7	0,02106	0,04307	0,03820	0,02743	——	98,48	98,51	90,82	90,02	88,91	96,87	95,83	96,09	85,98	84,13
S8	0,03158	0,04964	0,04244	0,03601	0,01267	——	97,29	91,65	90,36	85,77	97,17	96,08	95,96	87,92	86,04
S9	0,03835	0,04099	0,05138	0,03595	0,02321	0,02538	——	91,93	93,40	88,45	96,74	94,87	96,41	86,75	84,55
P1	0,13269	0,14404	0,14822	0,14138	0,12337	0,12717	0,12424	——	94,85	85,41	89,69	88,79	87,31	88,43	85,1
P3	0,14445	0,15267	0,15678	0,13530	0,13722	0,14360	0,12670	0,06485	——	90,33	88,61	89,54	90,08	87,88	87,38
P8	0,21853	0,21763	0,21811	0,20355	0,20614	0,21530	0,19177	0,13581	0,10495	——	83,33	86,33	85,26	85,07	86,51
F1	0,04099	0,06281	0,05813	0,05190	0,05376	0,05375	0,06444	0,12830	0,13507	0,20182	——	94,91	95,14	85,83	84,69
F2	0,04519	0,06267	0,05373	0,05189	0,05384	0,06014	0,06665	0,14180	0,14549	0,20393	0,03107	——	95,81	87,60	84,68
F3	0,04732	0,05574	0,05420	0,05187	0,06289	0,06280	0,07144	0,13962	0,14265	0,21558	0,04620	0,04186	——	87,05	84,14
Hi	0,18923	0,20444	0,18992	0,18649	0,17269	0,18409	0,17737	0,15153	0,15466	0,18822	0,19047	0,19927	0,20943	——	93,85
Ha	0,20463	0,22246	0,21008	0,20182	0,19277	0,20417	0,19736	0,16715	0,17270	0,20204	0,20987	0,21659	0,22251	0.04449	——

The European S-isolates and F-isolates are very similar compared to P-isolates. European P-isolates are much closer to each other than to North American P-isolates. The two Australian isolates are distantly related to the S, P & F-isolates of H. annosum. With the two Australian isolates, H. insulariae and H. araucariae, the percentage identity is 93.50%. The distance matrix data (Table. 2) also support the results.

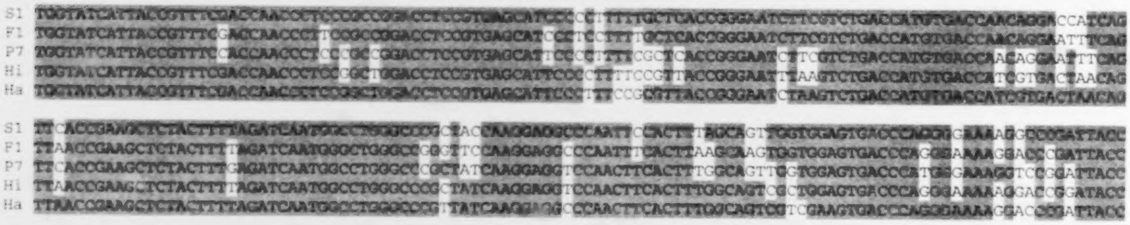

Fig. 1a. Alignment of nucleotide sequences of the laccase gene regions of isolates of S1 (S-type), F1 (F-type), P7 (P- type) of *Heterobasidion annosum*, Hi (*Heterobasidion insulare*), Ha (*Heterobasidion araucariae*). The sequences were aligned using Sequence Navigator. Variations in the nucleotide sequences in all the laccase are indicated by light background.

RESULTS

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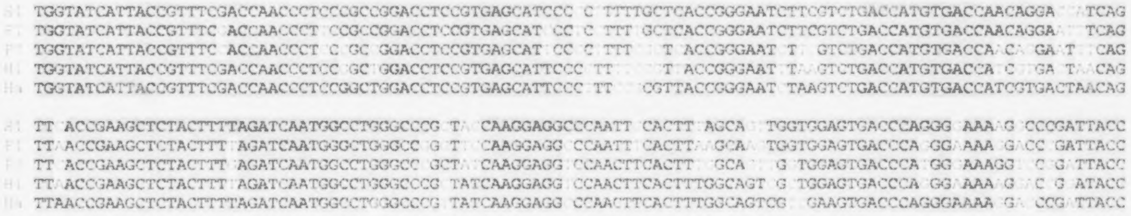
Strain code	S1	S3	S5	S6	S7	S8	S9	P1	P3	P8	F1	F2	F3	Hi	Ha
S1	-----	99.78	99,54	99,34	97,63	97,26	96.95	91,04	90,62	87,55	97,62	97,22	97,77	86.24	84,75
S3	0,03207	------	98,57	99,14	97,39	96,63	96,30	89.81	90,33	87,55	97,96	96,79	98,03	87,39	84.01
S5	0,02954	0,03881	------	97,71	96,23	95,86	95,69	89.51	90,75	87,22	96,26	96,03	97,71	86,25	86.15
S6	0,00639	0,01292	0,03000	-------	96,45	96,46	95,83	89,39	90,85	87,87	96,68	96,23	97,45	85,12	82,92
S7	0,02106	0,04307	0,03820	0,02743	-----	98,48	98,51	90.82	90,02	88,91	96,87	95,83	96,09	85,98	84.13
S8	0,03158	0,04964	0,04244	0,03601	0,01267	-------	97,29	91,65	90,36	85,77	97,17	96,09	95,96	87,92	86,04
S9	0,03835	0,04099	0,05138	0,03595	0,02321	0,02538	--------	91,93	93,40	88.45	96.74	94,87	96.41	86,75	84,55
P1	0,13269	0,14404	0,14822	0,14138	0,12337	0,12717	0,12424	-------	94,85	85,41	89,69	88,79	87,31	88,43	85,1
P3	0,14445	0,15267	0,15678	0,13530	0,13722	0,14360	0,12670	0.06485	------	90,33	88,61	89,54	90,08	87,88	87,38
P8	0,21853	0,21763	0,21811	0,20355	0,20614	0,21530	0,19177	0,13581	0,10495	-------	83,33	86,33	85,26	85,07	86,51
F1	0,04099	0,06281	0,05813	0,05190	0,05376	0,05375	0,06444	0,12830	0,13507	0,20182	------	94,91	95,14	85,83	84,69
F2	0,04519	0,06267	0,05373	0,05189	0,05384	0,06014	0,06665	0,14180	0,14549	0,20393	0,03107	-------	95,81	87,60	84,68
F3	0,04732	0,05574	0,05420	0,05187	0,06289	0,06280	0,07144	0,13962	0,14265	0,21558	0,04620	0,04186	-------	87,05	84,14
Hi	0,18923	0,20444	0,18992	0,18649	0,17269	0,18409	0,17737	0,15153	0,15466	0,18822	0,19047	0,19927	0,20943	-------	93,85
Ha	0,20463	0,22246	0,21008	0,20182	0,19277	0,20417	0,19736	0,16715	0,17270	0,20204	0,20987	0,21659	0,22251	0,04449	-----

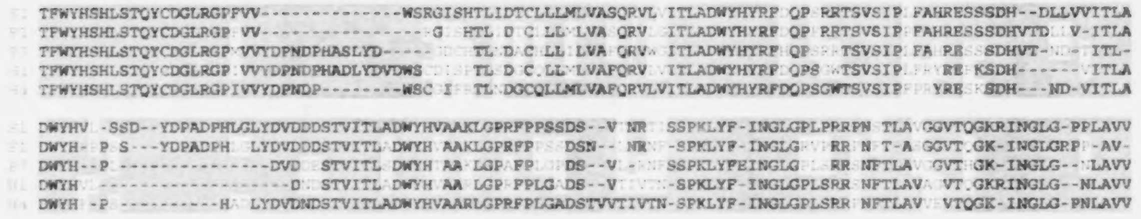
The European S-isolates and F-isolates are very similar compared to P-isolates. European P-isolates are much closer to each other than to North American P-isolates. The two Australian isolates are distantly related to the S, P & F-isolates of H. annosum. With the two Australian isolates, H. insulariae and H. araucariae, the percentage identity is 93.50%. The distance matrix data (Table. 2) also support the results.



```
S1 TGGTATCATTACCGTTTCGACCAACCCTCCCGCCGGACCTCCGTGAGCATCCC C TTTTGCTCACCGGGAATCTTCGTCTGACCATGTGACCAACAGGA   TCAG
F1 TGGTATCATTACCGTTTC ACCAACCCT CCGCCGGACCTCCGTGAGCAT CC C TTT GCTCACCGGGAATCTTCGTCTGACCATGTGACCAACAGGA   TCAG
P7 TGGTATCATTACCGTTTC ACCAACCCT C GC GGACCTCCGTGAGCAT CC C TTT   T ACCGGGAAT T  GTCTGACCATGTGACCA C  GA T  CAG
Hi TGGTATCATTACCGTTTCGACCAACCCTCC GC GGACCTCCGTGAGCATTCCC TT     T ACCGGGAAT T GTCTGACCATGTGACCA C  GA T  CAG
Ha TGGTATCATTACCGTTTCGACCAACCCTCCGGCTGGACCTCCGTGAGCATTCCC TT   CGTTACCGGGAAT TAAGTCTGACCATGTGACCATCGTGACTAACAG

S1 TT ACCGAAGCTCTACTTTTAGATCAATGGCCTGGCCCG T  CAAGGAGGCCCAATT CACTT AGCA  TGGTGGAGTGACCCAGGG AAA G CCCGATTACC
F1 TT ACCGAAGCTCTACTTT AGATCAATGGGCTGGCC G T  CAAGGAGG CCAATT CACTT  GCA  TGGTGGAGTGACCCA GG AAA G CC GATTACC
P7 TT ACCGAAGCTCTACTTT AGATCAATGGCCTGGCC GCT  CAAGGAGG CCAACTTCACTT  GCA  G TGGAGTGACCCA GG AAAGG  C G TTACC
Hi TT ACCGAAGCTCTACTTT AGATCAATGGCCTGGCCCG TATCAAGGAGG CCAACTTCACTTTGGCAGT G TGGAGTGACCCA GG AAA G  C G ATACC
Ha TTAACCGAAGCTCTACTTTTAGATCAATGGCCTGGCCCG TATCAAGGAGG CCAACTTCACTTTGGCAGTCG  GAAGTGACCCAGGGAAAA G  CCG TTACC
```

Fig. 1a. Alignment of nucleotide sequences of the laccase gene regions of isolates of S1 (S-type), F1 (F-type), P7 (P- type) of *Heterobasidion annosum*, Hi (*Heterobasidion insulare*), Ha (*Heterobasidion araucariae*). The sequences were aligned using Sequence Navigator. Variations in the nucleotide sequences in all the laccase are indicated by light background.



```
TFWYHSHLSTQYCDGLRGPFVV---------------WSRGISHTLIDTCLLLMLVASQRVL ITLADWYHYRF QP RRTSVSIP FAHRESSSDH--DLLVVITLA
TFWYHSHLSTQYCDGLRGP VV-----------------G  HTL D C LL LVA QRV  ITLADWYHYRF QP RRTSVSIP FAHRESSSDHVTD  V-ITLA
TFWYHSHLSTQYCDGLRGP VVYDPNDPHASLYD-----    TL D C LL LVA QRV  ITLADWYHYRF QP   TSVSIP FA RE  SDHVT-  - ITL-
TFWYHSHLSTQYCDGLRGP VVYDPNDPHADLYDVDWS     TL D C.LL LVAFQRV  ITLADWYHYRF QPS  TSVSIP    RE KSDH------ ITLA
TFWYHSHLSTQYCDGLRGPIVVYDPNDP---------WSC I  TL DGCQLLMLVAFQRVLVITLADWYHYRFDQPSGWTSVSIP    YRE  SDH---ND-VITLA

DWYHV -SSD--YDPADPHLGLYDVDDDSTVITLADWYHVAAKLGPRFPPSSDS--V NR  SSPKLYF-INGLGPLPPRPN TLA GGVTQGKRINGLG-PPLAVV
DWYH- P S---YDPADPH  LYDVDDDSTVITL DWYH AAKLGPRFP   DSN-- NR  -SPKLYF-INGLG   RR N T-A GGVT.GK-INGLGRP -AV-
DWYH--P ---------------DVD  STVITL DWYH AA LGP FP   DS--V -   SSPKLYFEINGLG   RR NFTLA  GVT GK-INGLG-- LAVV
DWYH  -------------------D  STVITL DWYH AA LGP FPLG DS--V    -SPKLYF-INGLGPLSRR NFTLAV  VT.GKRINGLG--NLAVV
DWYH -P ----------H  LYDVDNDSTVITLADWYHVAARLGPRFPLGADSTVVTIVTN-SPKLYF-INGLGPLSRR NFTLAV  VTQGK-INGLG-PNLAVV
```

Fig. 1b. Alignment of amino acid sequences of laccase genes of S, P & F-types of *H. annosum, H. insulare* and *H. araucariae*. Gaps were introduced where necessary to optimize the alignment. Variations in the amino acid residues in all the laccases are indicated by light background.

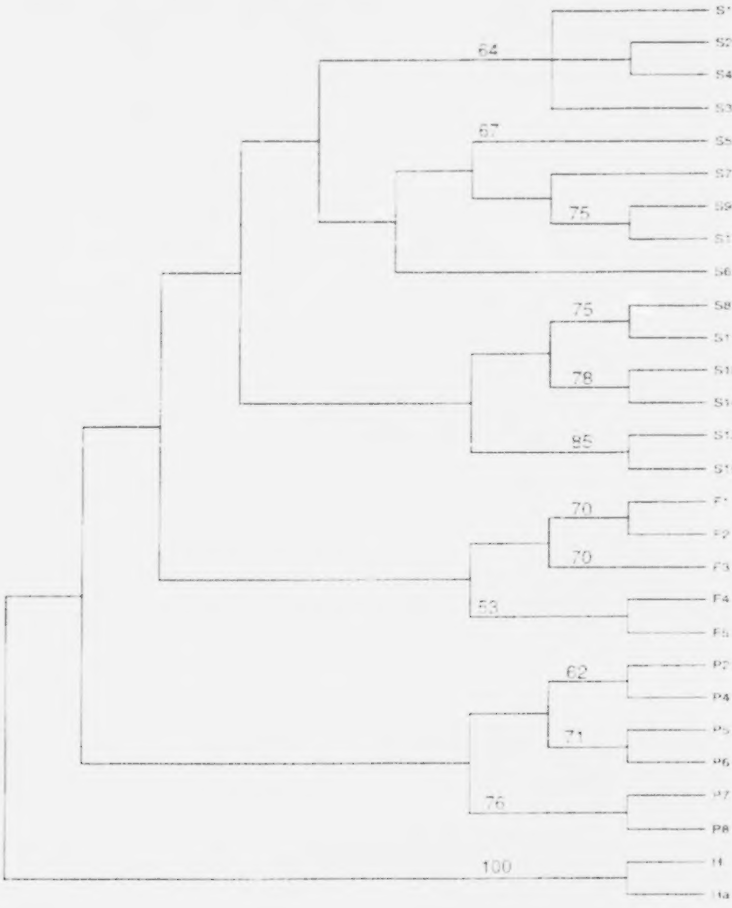


Fig. 2. Phylogenetic analysis of the laccase gene nucleotide sequences of different isolates of S, P and S-types of Heterobasidion annosum, H. insulare and H. araucaciae. Dendrogram represents the similarities between the different IGS group.

The European S-isolates are grouped together with a few North American S-isolates. Both North American and European P-isolates are grouped together and appear to be closely related to the European S-isolates (Fig. 2)

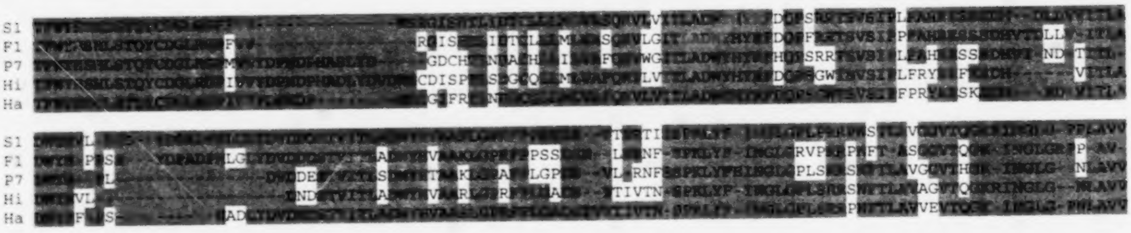

S1
F1
P7
Hi
Ha
S1
F1
P7
Hi
Ha

Fig. 1b. Alignment of amino acid sequences of laccase genes of S, P & F-types of *H. annosum, H. insulare* and *H. araucariae*. Gaps were introduced where necessary to optimize the alignment. Variations in the amino acid residues in all the laccases are indicated by light background.

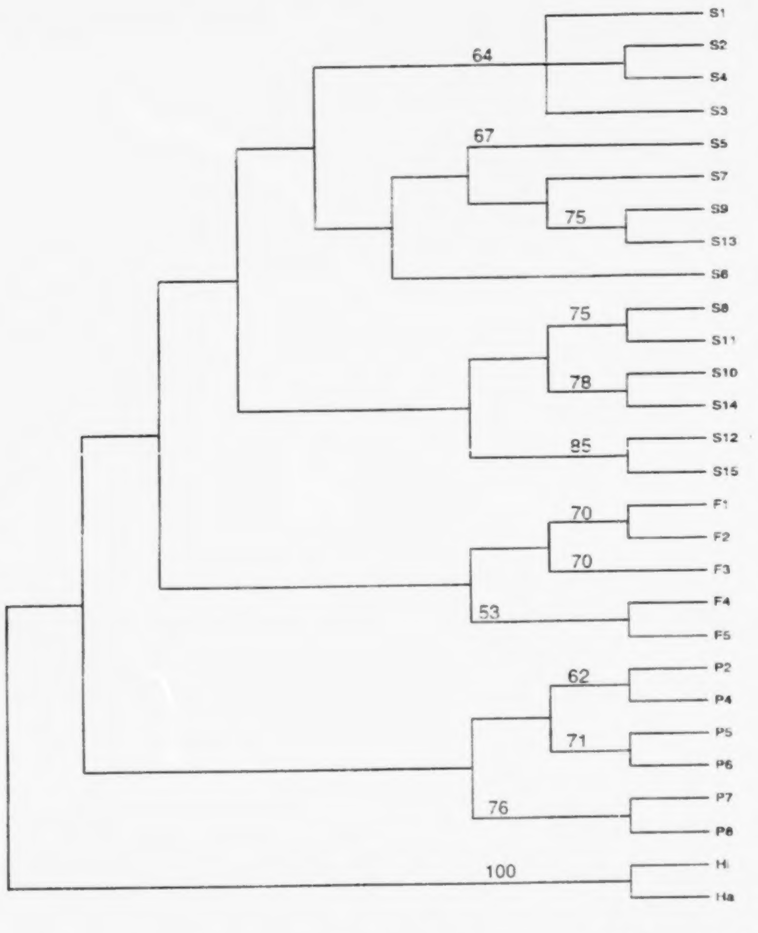

S1
S2
S4
S3
S5
S7
S9
S13
S6
S8
S11
S10
S14
S12
S15
F1
F2
F3
F4
F5
P2
P4
P5
P6
P7
P8
Hi
Ha
64
67
75
75
78
85
70
70
53
62
71
76
100

Fig. 2. Phylogenetic analysis of the laccase gene nucleotide sequences of different isolates of S, P and S-types of Heterobasidion annosum, H. insulare and H. araucaciae. Dendrogram represents the similarities between the different IG,S group.

The European S-isolates are grouped together with a few North American S-isolates. Both North American and European P-isolates are grouped together and appear to be closely related to the European S-isolates (Fig. 2)

DISCUSSION

We have observed that the sequence alignment of laccase genes of *H. annosum* IGs strains, *H. insulare,* and *H. araucariae* were consistent with the existence of different subgroups in this species. Previous authors noted that the S and F-types of *H. annosum* could not be distinguished by ITS or IGS sequences, yet RAPD markers have been used to delineate the types (La Porta et al. 1994), and there appear to be some differences in isozyme markers (Karlsson and Stenlid 1991; Otrosina et al. 1993). This study demonstrated the presence of additional polymorphisms within each subgroup and revealed some subgroups, one of them showing significant divergence, especially strain P8. Though the P-forms are represented by separate lineages, they appear similar ecologically. Unlike the F-form, the P-forms are pathogenic to even mature *Pinus sylvestris,* as well as other *Pinaceae*, other conifers and even hard wood species (Harrington et al. 1989; Korhonen 1978; Stenlid and Swedjemark 1998; Swedjemark and Stenlid 1995; Worrall et al. 1983). The occurrence of additional heterogeneity is consistent with earlier genotypic and phenotypic observations. Specifically, sequence heterogeneity was observed among genotype *H. insulariae* and *H. araucariae.* The sequences of *H. insulare* are closer than those of *H. araucariae* to *H. annosum* and also the pore width of the basidiome of *H. insulare* and the European P-type of *H. annosum* have been shown to be similar (Hood 1985). These results also match with the distance matrix data. Phylogenetic analysis separates the different S, P & F-isolates of *H. annosum, H. insulariae, and H. araucariae.* Furthermore, isozyme patterns of the American and European P form have been shown to differ markedly (Otrosina et al. 1993). These results suggest that there is a close relationship between the various IGs with respect to laccase genes. A long term goal and an important task are to gain a deeper understanding of the genetic differences occurring within and among the IGs.

ACKNOWLEDGEMENTS

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GENETIC VARIATION IN *HETEROBASIDION ABIETINUM* (*H. ANNOSUM* F GROUP) POPULATION

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SUMMARY

Isolates of *Heterobasidion abietinum* (*H. annosum*, F group) collected in different European countries, mostly from *Abies alba*, which is considered the typical host species, were recently studied. Amplification profiles obtained in PCR experiments using M13 minisatellite core sequence as primer were analysed and compared. The electrophoretic patterns of the fungal population exhibited a certain degree of geographical variation. This was probably related to the evolutionary movement of *A. alba* across the continent and accords well with the postglacial historical events of this conifer in the Mediterranean basin.

INTRODUCTION

Among the *Heterobasidion* species (*H. annosum, H. parviporum, H. abietinum* formerly *the* P, S and F groups of *H. annosum*) (Niemela and Korhonen 1998), the *H. abietinum* population has been little investigated, except for La Porta et al. (1998), who studied the Italian population. Consequently, little is known about this organism, which is widespread in the Mediterranean area, and about the possible relationship between the geographical distribution of this fungus in Europe and its genetic differentiation.

Abies alba Mill., the main host species, has, like *H. abietinum* itself, its centre of distribution in the Mediterranean area (Korhonen et al. 1998). Both the fungal pathogen and the host tree have shared the same territory for a long time, at least since the post-glacial period, when the *Abies* range was extremely reduced to the southern end of the Mediterranean peninsulas (Bernetti 1998).

The aim of this study was to examine the genetic variations of *H. abietinum* collected in different European countries in relation to the evolutionary movement of its typical host, *A. alba*.

MATERIALS AND METHODS

H. abietinum isolates studied come from different geographical areas: the alpine area (Italy, Austria, Bulgaria, Poland, Switzerland and Slovenia) and the Mediterranean basin (Italian Apennines, Greece and the Pyrenees). DNA was extracted from fungal cultures using the procedure of Smith and Stanosz (1995). Isolates of *H. annosum ss* and *H. parviporum* were included in the test for comparison. Amplification profiles obtained in PCR experiments using the M13 minisatellite core sequence (Karlsson 1993) were analysed and compared. Amplification products were used to create a binary absence/presence (0/1) matrix, used for statistical analysis. The genetic similarity coefficients (DICE) were calculated according to Gabriel et al. (1988). Phylogenetic dendrograms were constructed from the DICEs by SAHN clustering (UPGMA method). Clustering analysis and principal components analysis based on the NTSYS-pc program (Rohlf 1987).

RESULTS AND CONCLUSION

Comparison of the different *Heterobasidion* isolates showed that variation was higher between groups than between populations or individuals. The *H. abietinum* isolates from the Alpine regions exhibited very little variation between geographical groups, while variation among and within populations was higher. Isolates from Italian Apennines, the Greek peninsula and the Pyrenees exhibited a greater variation between groups than between populations. The genetic difference among *H. abietinum* isolates from the Italian, Greek and Iberian peninsulas underline the limited genetic flow of this fungus, which was due to the long distances between these areas (Fig. 1). Consequently, each population changed in different ways independently of the others, modifying a genetic heritage that was probably the same throughout before glaciation. The fundings accord well with the postglacial movements of *Abies* in Europe and underline that the pathogen population was genetically influenced by the same factors as the host tree.

In the Alps and central Europe, where differentiation between *A. alba* ecotypes was not found, pathogen variation was also slight (Fig. 2). In this study, individual differences between *H. abietinum* isolates were higher than variations between groups and populations, probably because the long distances between the collection areas meant that the genetic flow was low.

After the last glaciation, only a few *Abies* populations survived in the Dinarich Alps, the Carpathian Mountains and southern Russia (Schmidt-Vogt 1977). *A. alba* found refuge in Calabria (southern Italy), the Balkan region and the Pyrenees. This species began the re-colonisation of its present area moving from south to north and from west to east in the Alps; the present diffusion of *A. alba* in central Europe is due to the spread of the Italian populations' expansion movements (Bernetti 1998).

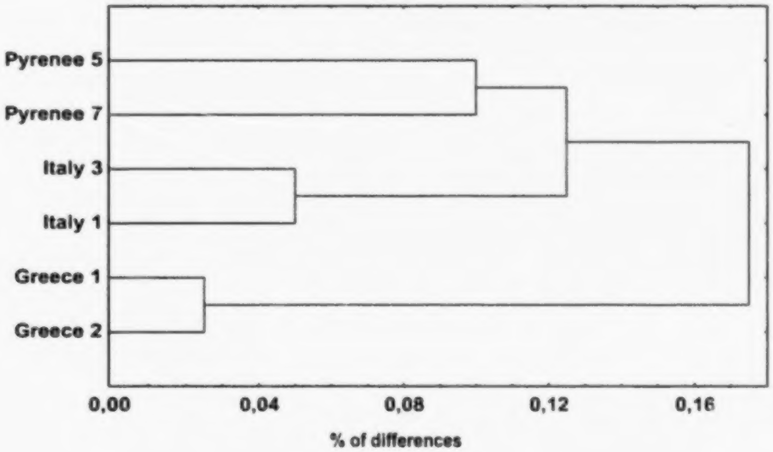

Fig. 1 UPGMA cluster dendrogram of a selection of *Heterobasidion abietinum* isolates collected from different mediterranean countries.

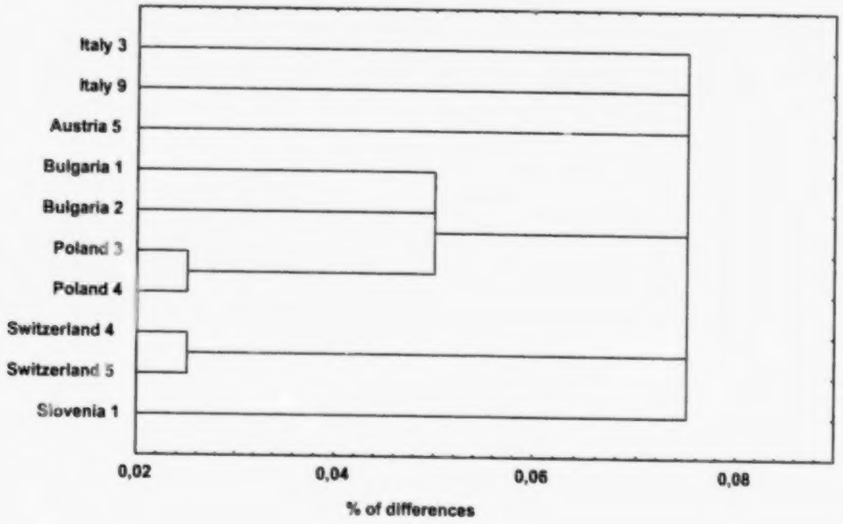

Fig. 2 UPGMA cluster dendrogram of a selection of *Heterobasidion abietinum* isolates collected from Alpine and central Europe.

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APPLICATION OF GENETIC MARKERS FOR BIOLOGICAL STUDIES OF *ARMILLARIA*

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Genetic markers generated by amplified fragment length polymorphisms (AFLP) and intergenic spacer region (IGS) sequence polymorphisms are being used to characterize *Armillaria* species and genets. Ecological behavior (e.g., pathogenic or saprophytic) of *Armillaria* genets and associated environmental data are being recorded from the *Armillaria* collection sites. Each collection site will be verified for Potential Vegetation Group (PVG or subseries). In addition, some collection sites will be monitored for environmental attributes (host, soil properties, moisture, temperature, light, etc.). Genetic markers will be analyzed for relationships to environmental attributes and ecological behavior at the species and genet level. This approach may allow an interpretation of environmental adaptation and gene flow among *Armillaria* genets and populations. These studies should also help define critical environmental variables that determine distribution of saprophytic and pathogenic *Armillaria* spp. The integration of genetic marker data with geographic information systems (GIS) may allow prediction of *Armillaria* distribution and behavior at the landscape level. Information derived from these studies should provide new tools and approaches to manage Armillaria root disease.

IDENTIFICATION OF PATHOGENICITY GENES IN *HETEROBASIDION ANNOSUM* USING EXPRESSED SEQUENCE TAGS (ESTs)

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The basidiomycete fungus *Heterobasidion annosum* is the causal agent of annosum root rot and is economically the most important disease of coniferous forests in northern temperate regions. *H. annosum* is a wood-decaying pathogen which can utilize a variety of carbon sources, such as starch, cellulose and other glucans, pectin, lignin and various phenolic compounds. It is known to secrete a wide range of extracellular enzymes and toxins. The purpose of this research project is to investigate the pathogenicity of *H. annosum* and to identify key factors that enable the fungus to infect and cause disease. A cDNA-library was constructed from *H. annosum* mycelia, where pathogenicity genes were induced by *Pinus sylvestris* seedling roots. Partial sequencing of individual cDNA-fragments yields expressed sequence tags (ESTs). The results from the first sequencing indicate that the cDNA-library is of good quality with fragment lengths spanning from 350 bp to 2500 bp with an average of 900 bp. So far about 1000 clones have been sequenced. The expressed tags are used in homology searches against available sequence information in public databases. This will give an idea of the genes expressed during the infection process. So far, 27% of the sequences do not show any significant homology to characterized genes, while 40% seem to be involved in protein synthesis, and 33% show significant homology to other genes. In order to identify genes that are important for the pathogenicity of the fungus, the cDNA fragments will be screened for differential expression, comparing induced and control mycelia. A further study of putative pathogenicity genes will include extensive expression profiling.

POPULATION STRUCTURE OF *ARMILLARIA* SPP. AND *MEGACOLLYBIA PLATYPHYLLA* IN *QUERCUS RUBRA* STUMPS OVER A 17-YEAR PERIOD

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Biotic or abiotic stress factors may enable several *Armillaria* species to become aggressive secondary pathogens of forest trees. One stress agent causing expensive defoliation in eastern North American oak forests is the gypsy moth (*Lymantria dispar* L.). Increased mortality in these stands following defoliation is often caused by *Armillaria*. There is some concern among forest managers that cuttings resulting in large stumps may provide more inoculum potential for *Armillaria* since saprobic activity is essential to its success in gaining resources (Hood et al. 1991). Some cord-forming fungi closely resemble *Armillaria* in behavior, except for their lack of pathogenicity (Rayner 1977). Some of these fungi may function as biocontrol organisms by preventing rhizomorph growth or mycelial development, limiting the pathogen to its own occupied substrate, or perhaps by eliminating *Armillaria* from substrate already occupied. One such organism is *Megacollybia platyphylla,* native to North America from Québec to Florida and east to Iowa. Several studies suggest *Armillaria* and *M. platyphylla* compete for substrates. In this study, we examined the effect of time since cutting on the sequence and position of both of these fungi in red oak stumps and roots. Our data show that *M. platyphylla* and *Armillaria* spp. compete for resources over time, with *M. platyphylla* increasing in abundance, while *Armillaria* decreases. Thinning stands in advance of an anticipated gypsy moth defoliation may allow *M. platyphylla* to compete with *Armillaria* for downed woody resources and stumps, thus acting as a biocontrol agent of *Armillaria*. Whether or not the decay process can be accelerated by artificial inoculation remains unclear.

PRESENCE OF DSRNA IN *HETEROBASIDION ANNOSUM*

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A search for double-stranded RNA (dsRNA) was conducted among 204 European isolates of the pathogenic fungus *Heterobasidion annosum*. Nucleic acids were extracted and purified by cellulose CF-11 chromatography or lithium chloride precipitation. dsRNA was present in eight of the isolates and was confirmed by nuclease digestion. The dsRNA elements ranged between 1.8 and 2.4 kbp and were found in two of *H. annosum* intersterility groups, S and P. Partial amino acid sequence information from one dsRNA element showed significant homology to RNA dependent RNA polymerases from several fungal partitiviruses. This is the first report of the presence of dsRNAs in *H. annosum*. Possible implications of dsRNA for the biology of the fungus and the potential for biological control are discussed.

POPULATION STRUCTURE OF TWO *ARMILLARIA* SPECIES COEXISTING IN MANAGED MOUNTAINOUS NORWAY SPRUCE FORESTS

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SUMMARY

The preferentially saprotrophic *A. cepistipes* is the most common *Armillaria* species in Swiss spruce forests and could have a potential to be used against the pathogenic *A. ostoyae* or other root and butt rot fungi. The objective of this study was to determine the population structure of *A. cepistipes* and *A. ostoyae* when they coexist in managed spruce forests.

The three plots (1 ha) were located in naturally regenerated managed Norway spruce (*Picea abies*) stands at approximately 1400 m a.s.l. In each plot, rhizomorphs were collected on a regular sample grid (10x10 m) and stumps were examined for the presence of mycelial fans.

All the plots were characterized by the presence of two to six genets of each *Armillaria* species. Intra- and interspecific spatial overlap were rare except in one plot. Generally, stumps were colonized by the genet which also occurred in the nearby soil. The presence of two genets on the same stump was observed only on the border between genets. The results provide evidence for intra- and interspecific competition between *A. cepistipes* and *A. ostoyae* and also suggest that these species occupy very similar ecological niches in spruce stands.

Pathogenicity, Resistance and Etiology

GENETIC VARIATION IN SUSCEPTIBILITY TO *HETEROBASIDION ANNOSUM* INFECTION IN SPORE-INOCULATED FRESH STUMPS OF *PICEA ABIES* CLONES

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SUMMARY

Fresh stumps were created by cutting 15-year-old *Picea abies* trees, whereupon the surface of each was immediately inoculated with a suspension of conodiospores from an isolate of the S-intersterility group of *Heterobasidion annosum*. In total, 50 different clones were represented among the 433 stumps included in the test, which was part of a clone trial situated in southern Sweden. The inoculation period extended from September to March.

The frequency of infection on the stump surface was 98%. The mean vertical distance of fungal growth in the stumps was 6 cm (0-40 cm), and 29% of the tested trees were shown to be naturally infected with *H. annosum*. Significant differences among clones in infection frequency (broad sense heritability H^2=0.27) and in vertical spread of the fungus in the stump (H^2=0.15) were found. The vertical extension of mycelium of the artificially inoculated fungus was greater in trees that already contained *H. annosum*.

The results indicate that the incidence of infection and mycelial extension of *H. annosum* in *P. abies* differ among individual trees following primary artificial conidiospore infection on stumps.

INTRODUCTION

Commercial clonal forestry programmes on Norway spruce (*Picea abies* (L.) Karst) have been under way in Sweden since 1975 (Karlsson 1993). Several investigations have demonstrated the existence of significant clonal variation in Norway spruce for traits such as growth capacity, growth rhythm and wood density (St. Clair and Kleinschmit 1986; Roulund et al. 1986; Shaw et al. 1988; Lepistö 1993; Karlsson and Högberg 1998). Knowledge about genetic variation and heritability (H^2) is essential in order to predict future selection gain (Zobel and Talbert 1984).

Among-clone variation in the vertical spread of the root and butt rot fungus *Heterobasidion annosum* (Fr.) Bref. in the stem of cuttings and young trees of Norway spruce has been detected in inoculation experiments (Weissenberg 1975; Dimitri and Schumann 1989; Swedjemark and Stenlid 1996; 1997; Swedjemark et al. 1997). In the last-mentioned investigation, 35% of the variation was explained by the genetic constitution of the host. Individual stumps of Norway spruce and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) have been shown to be more or less susceptible to *H. annosum* spore infections (Redfern 1993; Redfern et al. 1997; Bendz-Hellgren 1997). Differences were detected on both artificially and naturally infected stumps.

H. annosum is a serious pathogen in coniferous forests throughout the boreal and temperate zones of the Northern Hemisphere. It can rapidly destroy a stand under optimal conditions (Swedjemark and Stenlid 1993). Decay in attacked trunks may spread up to 12 m height (Stenlid and Wästerlund 1986), and lateral spread in a stand has been reported to be up to 70 cm per year (Swedjemark and Stenlid 1993). Economic losses are due to reductions in both the quality and production of wood (Bendz-Hellgren and Stenlid 1995; 1997). Spores land on recently exposed wood, such as freshly cut stumps, germinate and form a mycelium which colonises the stump (Rishbeth 1951a). The fungus then extends throughout the roots and infects healthy trees via root contacts between stumps and trees (Rishbeth 1951b). In nature, infection is initiated by basidiospores germinating and establishing a heterokaryotic mycelium in the stump. Although conidiospores have been noted in nature, their role in the infection of stumps is not yet clear (Hsiang et al. 1989). Heterokaryotic mycelium are generally believed to be more pathogenic than homokaryotic ones, even though homokaryotic mycelium have been found in living white fir (*Abies concolor* (Gord. Glend.) Lindl.) (Garbelotto et al. 1997) and in Norway spruce stumps four months after felling (Stenlid 1994). However, if the spore load is heavy enough conidiospores can form a heterokaryotic mycelium by merging two homokaryotic mycelia. The probability of infection success increases with the number of spores deposited on the stump (Van der Plank 1975) as long as no intraspecific competition occurs (Redfern et al. 1997). To avoid competition between fungal individuals, conidia from a single *H. annosum* isolate can be used for inoculation. In previous inoculation experiments high density loads of conidiospores have been used (up to 600 000 spores per inoculation). This does not correspond with natural conditions, where the spore load is much smaller. Rates of natural spore deposition recorded range between 0.59 and 1932 spores /dm^{-2} h^{-1} (Rishbeth 1959; Sinclair 1964; Kallio 1970; Edmonds and Driver 1974; Edmonds et al. 1984a, b). Kuhlman and Hendrix (1964) found that a suspension of basidiospores colonised about the same volume of *Pinus echinata* (Mill.) stump wood as a 600-fold suspension of conidiospores did.

Economic losses could be reduced substantially if plant material less susceptible to growth of *H. annosum* in the wood could be used since spread of the fungus in the stand would be reduced, and less wood would be destroyed per infected tree. If one could find clones whose resistance mechanisms remain intact in the stump for some time after felling, the gain could be even greater.

The aim of this study was to determine if there are any differences among Norway spruce clones in terms of the fate of conidiospores of *H. annosum* landing on freshly cut stumps. Two hypotheses were tested: (1) There is a difference in stump susceptibility to infection by *H. annosum* spores among clones, and (2) the growth rate of the established mycelia of *H. annosum* in the stump differ among clones.

MATERIAL AND METHODS

The experiment was set up in a second-generation, 14-year-old, unthinned Norway spruce clone test. The test consisted of cuttings of 311 clones and was located on the Hjuleberg estate in south-western Sweden. Cuttings used in the trial were planted with a 1.4-m spacing according to a Roman square design with single-tree plots, but in the analysis it was regarded as a randomised block experiment. The trial (no. 979) was part of a Swedish regional clonal forestry programme, described in more detail in Karlsson and Högberg (1998). No signs of *H. annosum* infection had previously been observed in the trial.

In September 1997, 50 different clones were randomly selected from among the 273 clones that had at least eight out of nine ramets remaining in the trial. No bias should have been added by selecting clones with higher survival rate since there was no significant difference in mortality among the clones (Karlsson and Högberg 1998). In total, 433 trees were felled, leaving stumps 60-70 cm high. Immediately after felling, the stumps were inoculated with a suspension of *H. annosum* conidiospores originating from one isolate of the S-intersterility-group. This fungal isolate (Rb 175) was obtained in 1985 from a living Norway spruce in southern Sweden (Stenlid 1987) and has been used in several inoculation experiments (Swedjemark and Stenlid 1994; 1995; 1996; 1997; Swedjemark et al. 1997). The isolate was incubated on agar-medium in petri-dishes which were flooded with 100 ml of sterile water immediately before inoculation. Using a haemocytometer, we determined that the suspension contained ca 8000 spores per ml. Each stump was inoculated with a density of ca 80 spores per cm^2 stump surface. The stumps were not covered after inoculation, thereby allowing natural infection as well.

The first assessment was conducted in November 1997. A 2-cm-thick disc was cut from each stump, marked with identity and position of the tree from which it was taken, and transported to the laboratory in a plastic bag. The discs were then incubated at 20°C for about 7 days, whereupon they were examined to determine the conidial stage of *H. annosum*.

In the spring of 1998, the extension of the fungus in the stumps was recorded. Each stump (60 – 70 cm long) was cut 5 cm above ground and brought to the laboratory, where it was divided into three equal parts. The upper part was cut into ten discs (2 cm thick), the middle part into four discs (5 cm), and the bottom part into two or three discs (10 cm). The discs were numbered and put separately into plastic bags which were incubated at 20°C for 7 days until checked for conidiophores of *H. annosum*. Any visible decay in the bottom part of the stump was noted. For practical reasons, the stumps were felled on four occasions: March 9, April 8 and 24, and May 13. Due to technical problems, three clones were lost in connection with the second assessment.

The scored traits with few classes departed from a normal distribution and these variables were transformed to normal scores before analysis (Gianola and Norton 1981).

Estimates of the variance components with their standard errors for estimation of quantitative genetic parameters were obtained assuming the following model:

$$Y_{ijk} = \mu + B_i + c_j + e_{ijk} \qquad [1]$$

where:
Y_{ijk} = value of the ijk^{th} observation
μ = mean value of the population
B_i = fixed effect of block i
c_j = random effect of clone j $(N\sigma^2_c)$
e_{ijk} = random error term $(N\sigma^2_e)$

Genetic parameters were interpreted as:

$$\sigma^2_G = \sigma^2_c,\ \sigma^2_E = \sigma^2_e \qquad [2]$$

where:
σ^2_G = the genotypic variance
σ^2_E = the environmental variance

Individual tree broad sense heritabilities (H^2) were calculated as the ratio between σ^2_G and $\sigma^2_G + \sigma^2_E$ (Falconer 1989; Becker 1992).The analyses were carried out using the Proc Mixed in the SAS software (SAS 1996), which is based on mixed model equations (MME) and the restricted maximum likelihood method (REML). Z-tests were carried out to test whether the variance components were statistically significant different from zero ($p<0.05$). Best Linear Unbiased Predictors (BLUP) were obtained for the clones using the same SAS procedure. Product-moment correlations were estimated between pairs of BLUP-values using Proc Corr (SAS Institute Inc., Cary, NC, USA).

RESULTS

First assessment

The incidence of infection was 98%. The diameter was positively correlated with infection frequency. The diameter of the stumps ranged between 1.5 cm and 13 cm, with a mean value of 8 cm (Tab. 1). The incidence of infection and the diameter of the stumps differed significantly among clones, and the H^2 for incidence of infection was 0.27 (Tab. 2).

Second assessment

The mean vertical distance of fungal spread was 5.8 cm, and values differed significantly among clones, ranging between 0 and 40.0 cm (Table 1, 2). The mean over all clones ranged between 4.6 cm and 6.7 cm and did not differ significantly among felling occasions. H^2 was 0.15 for vertical growth of *H. annosum* (Table 2). Natural infections of *H. annosum* were found in 29% of the stumps, but there was no significant difference among clones or among blocks. Infection frequency and vertical fungal growth showed significant phenotypic correlations with growth parameters (height, increment and diameter). Fungal extension was also correlated to the occurrence of natural infection (Table 3). Estimated correlations between BLUP-values for infection frequency and growth parameters were significant (Table 3).

DISCUSSION

There was significant variation among clones in terms of the susceptibility of stumps to the conidiospores of *H. annosum*, even though the proportion of uninfected stumps was low. Redfern et al. (1997) found that some Sitka spruce stumps seemed to be more resistant to *H. annosum* basidiospore infection than other stumps in the same stand. The uninfected proportion of stumps was largest among those groups receiving natural or low artificial spore loads (89-560 basidiospores/ml), but some stumps remained uncolonised even after inoculation with higher spore doses (5000 - 580 000 basidiospores/ml). A suspension of basidiospores is presumed to have an infective capacity about 600 times stronger than of a suspension of conidiospores of the same concentration (Kuhlman and Hendrix 1964). In an experiment with 300 Norway spruce stumps incoculated with suspension containing 400 conidiospores/ml, Bendz-Hellgren (1997) found significant differences in susceptibility between stumps. In our study, the inoculant contained about 8000 conidiospores/ml. Thus, the proportion of uninfected stumps might have been lower if a lower load of spores had been used. However, we were anxious to obtain a high infection rate in order to optimise the results of the vertical growth analysis of the pathogen in the stump.

A freshly cut stump offers a special set of substrate conditions since it is not a living tree, nor is it a dead substrate. Rather, it consists of tissue that remains metabolically active for some time after felling of the tree. Meredith (1959) showed that saprophytic fungi do not invade stump roots during the first few months after felling, which indicates that the resistance mechanisms of the felled tree are still, at least partly, intact. Pathogenic fungi, such as *H. annosum*, can therefore establish themselves and colonise the stump without any interference from saprophytic competitors. The period during which the stump stays active will vary depending on several factors, including tree species, stand density, stump size, macroclimate and microclimate. Several of these factors have been shown to be important in determining the extent of colonisation of stumps (Yde-Andersen 1962; Gooding et al. 1966; Shaw 1989; Redfern 1993; Bendz-Hellgren and Stenlid 1998). Our results suggest that resistance mechanisms in the individual clone may be an important factor affecting pathogen colonisation success even after felling.

Bendz-Hellgren et al. (1998) found significant differences in fungal growth rate between individual stumps in stands that were 30 to 40 years old, 1 to 3 years after inoculation with *H. annosum* mycelium. In this investigation there was a difference in fungal growth in the stumps after 4 to 6 months. If this difference is still present after 3 years, it may have a large impact on the spreading rate of the pathogen in an affected stand.

Dimitri and Schumann (1989) found that inoculated 8-year-old trees had excluded the fungus from the stem after one year. Swedjemark et al. (2001) found significant differences between inoculated 4-year-old cuttings in the ability to exclude the fungus from the stem after 6 months. If freshly cut stumps has the ability to exclude the pathogen still has to be investigated.

Twenty-nine percent of the trees were found to be naturally infected with *H. annosum*. Since the trees cut represented the second generation of spruce on the site and no thinning operations had been undertaken, the pathogen must have spread to these trees from the root systems of the previous stand (Stenlid 1987; Swedjemark and Stenlid 1993; Piri 1996; Rönnberg and Jörgenssen 2000). We were not able to detect any significant differences in natural infection frequency among clones, possibly owing to the limited number of naturally infected trees (116 trees). However, the percentage of rametes naturally infected with root rot was high for some clones (80%) but much lower (0% in some cases) for others. In a future study we plan to analyse a larger number of trees in the stand in order to get a more detailed natural infection pattern.

The artificial infection spread further in trees that contained natural *H. annosum* than in healthy trees. There are two conceivable explanations for this: **(1)** The root infection may have disturbed the functioning of trees, thereby increasing their susceptibility to pathogens. For instance, root infection can reduce water uptake, thus inducing drought stress in trees. Drought stress has been shown to increase susceptibility to *H. annosum* infection in *Pinus taeda* in both field and greenhouse tests (Tower and Stambaugh 1968). Similarly, in inoculation trials on seedlings and cuttings of Norway spruce, Lindberg and Johansson (1992) and Swedjemark and Stenlid (1997) found that drought stress and root health affected the predisposition to infection by *H. annosum*. However, in the present investigation no stress symptoms were observed, nor was there any decrease in increment in trees attacked by *H. annosum*. In addition, the infection frequency was higher and the vertical growth of the artificially inoculated fungus was longer in large, well-growing clones. These observations agree with the results of Wahlström and Barklund (1994), who found that the growth rate of *H. annosum* was higher in irrigated and fertilised 26-year-old spruces than in trees subjected to drought stress. **(2)** The affected clones were more susceptible to *H. annosum*. Based on the results from previous inoculation experiments with cuttings (Dimitri and Schumann 1989; Weissenberg 1975; Swedjemark and Stenlid 1996; 1997; Swedjemark et al. 1997) and results of the present investigation this explanation seems reasonable. Nevertheless, the non-significant genotypic correlation between frequency of natural root rot and the growth of spore-inoculated fungus does not support this explanation. In addition, the natural root-rot frequency measurements were biased since no effort was made to detect infections below the stump although they were likely to exist in many trees considering the high frequency of trees infected at stump level. However, vertical extension of the pathogen was greater in trees with detectable decay at stump level. Further resistance testing and a more extensive field analysis on the same clones will have to be made before any conclusions can be drawn in this regard.

From a genetic point of view the results of our study confirm findings from earlier studies with *H. annosum* inoculations in different clones of Norway spruce (Swedjemark and Stenlid 1996; 1997; Swedjemark et al. 1997): Fungal extension (or resistance to fungal growth) was affected by genotype to about the same degree as growth traits in this and other trials in the same series (Karlsson and Högberg 1998) as well as in other studies (Bentzer 1993; Cornelius 1994). The remaining question, however, is whether our results also apply to resistance to natural infection in forest stands and to the total loss of fibre in mature stands. If the juvenile-mature correlations would be significant, the genotypic parameters would be large enough to allow considerable gain through clonal selection and long-term breeding work.

Table 1. Mean values for infection frequency (%), Fungal vertical growth (mm), natural *H. annosum* infection from the previous generation spruce (%), height measured 1994 (dm) and diameter of the top disc (cm) for each clone. The clones are sorted on descending fungal growth value.

Clone	Infection freq. (%)	Fungal growth (mm)	Natural infect. (%)	Height-94 (dm)	Diameter (cm)
4468	100	13.8	78	59.4	8.8
2371	100	10.0	13	50.1	7.8
3535	100	9.9	44	59.2	10.7
2396	88	8.4	33	48.3	7.7
1332	100	7.4	33	60.7	9.5
4824	100	7.4	22	47.1	8.2
2071	100	7.3	25	54.1	8.0
3917	100	7.3	13	55.1	8.3
2382	100	7.2	33	41.4	6.9

1494	100	7.1	14	58.4	8.4
269	100	7.0	38	41.9	6.4
4876	100	7.0	33	56.8	7.8
7632	100	7.0	25	41.4	6.0
4162	100	6.8	14	48.6	6.5
2411	88	6.5	68	51.5	8.2
2228	100	6.3	29	61.9	9.7
8500	100	6.3	11	56.5	8.6
33	100	6.2	38	41.4	7.1
5600	100	6.1	38	54.0	8.3
8607	100	6.0	22	56.2	8.5
1591	100	5.8	11	49.8	7.9
7444	100	5.8	56	43.9	6.9
1779	100	5.7	22	55.6	8.3
2647	100	5.7	0	53.3	7.1
4979	100	5.7	33	44.7	5.9
7934	100	5.7	50	48.0	7.2
442	100	5.6	11	57.4	9.5
5342	100	5.2	38	55.2	9.9
1392	100	5.1	22	53.8	7.2
181	78	4.9	57	51.5	9.3
2443	100	4.8	33	45.7	7.3
5607	100	4.8	22	46.2	6.9
9137	57	4.7	38	39.2	5.8
219	100	4.6	11	50.6	8.2
8191	100	4.6	22	59.5	9.3
3090	100	4.5	33	36.1	5.5
1216	100	4.0	22	56.9	9.9
3799	100	4.0	22	59.2	10.1
1229	89	3.7	13	47.0	5.5
2259	100	3.6	50	38.6	4.9
4668	100	3.5	0	52.5	6.4
558	83	3.4	17	61.9	9.0
4148	100	3.4	38	60.8	9.0
1999	100	3.3	13	46.9	6.4
3161	100	2.7	38	59.1	9.5
2665	100	2.5	25	57.9	8.4
1450	100	2.0	29	52.5	7.2
42	100	.	0	.	11.5
72	100	.	25	.	9.0
3353	100	.	29	42.0	6.2
All	98	5.8	29	51.5	7.9

Table 2. Broad sense heritabilities (H^2) and significance levels for variance components $P(\sigma^2_G)$.

	H^2	$P(\sigma^2_G)$
Infection frequency	0.27	0.0004
Vertical fungal growth	0.15	0.0147
Natural root rot	0.01	0.8520
Stump diameter	0.32	0.0002
Height 1994	0.33	0.0001

Table 3. Significance levels for estimated correlation coefficients (upper right diagonal of the table) and phenotypic correlation estimates (lower left diagonal). NS = $p > 0.005$, * = $p < 0.05$, ** = $p < 0.0005$ and *** = $p < 0.0001$.

	Height 89	**Height 94**	**Increment**	**Diameter**	**Infection frequency**	**Vertical fungal growth**	**Natural infection**
Height 89		***	***	***	NS	NS	NS
Height 94	***		***	*	*	NS	NS
Increment	***	***		***	**	NS	NS
Diameter	***	***	***		*	NS	NS
Infection frequency (artificial infection)	***	***	***	***		NS	NS
Vertical fungal growth (art. inf.)	*	**	***	**	**		NS
Natural infection	NS	NS	NS	NS	NS	***	

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PATHOGENICITY OF P-, S-, AND F-INTERSTERILITY GROUPS OF *HETEROBASIDION ANNOSUM* TO SCOTS PINE, NORWAY SPRUCE AND COMMON FIR IN INOCULATION EXPERIMENTS

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SUMMARY

Infection experiments with *Heterobasidion annosum* isolates from P, S and F intersterility groups were conducted on pine and spruce seedlings grown *in vitro* and on four-year-old pine, spruce and fir trees, in the field and in a greenhouse. Despite the different measurements of the pathogen virulence, the study provided evidence for the existence of host preference in *H. annosum* complex. The strain and IS group effects accounted for the highest portion of the explained variation in seedling mortality rate and in the pathogen spread in stems, while the host-plant accounted for the smallest portion. Although statistically insignificant, the differences in the host preference showed by the IS groups in the greenhouse experiments showed similar tendency to that observed in the *in vitro* and field experiments.

Keywords: *Heterobasidion annosum*, intersterility groups, pine, spruce, fir, infection experiments

INTRODUCTION

Heterobasidion annosum (Fr.) Bref. is one of the most important specialized root rot pathogens in the temperate and boreal regions of the world causing a great losses to the timber production, mainly in managed forests. This species consists of three intersterility (IS) groups showing different preferences to the host trees (Korhonen 1978; Capretti et al. 1990).

Various inoculation methods were applied for study host resistance and virulence of *H. annosum*. Although seedlings younger than one year are considered to be useful to study resistance mechanisms, and consequently were used in studies on infection process and host-pathogen interaction (Asiegbu et al. 1993; Heneen et al. 1994, Werner and Idzikowska 2001), they were seldom used to test the host resistance and virulence of the pathogen. Taking mortality rate of pine and spruce seedlings as the measurement of susceptibility, a higher resistance of spruce was shown by Dimitri (1963). Contrasting results were obtained by Hüppel (1970), and recently by Werner and Łakomy (in press). Most of the infection experiments used direct wounding methods. Criteria for assessing the host resistance and pathogenicity of the fungus in these methods are the fungal spread in the wood and/or length of necrosis in the inner bark (Dimitri 1963; Delatour 1982; Stenlid and Swedjemark 1988; Swedjemark et al.1999). In the methods, trees in age from 2 to 100 years were used. Infection rate and extension of living mycelium in stems were usually higher in spruce than in pine. Methods for inoculation of roots, due to low infection frequency, are less successful (Rishbeth 1951, Boyce 1962).

The objectives of this study were (i) to compare different virulence of P-, S-, and F-group strains of *H. annosum* on three main host-plants using three different methods of inoculation; (ii) to estimate portions of host, strain and IS group contribution to the total variation.

MATERIALS AND METHODS

Inoculation experiments *in vitro*

The plant material consisted of *Pinus sylvestris* L. seedlings representing the provenance of Bolewice (52 °28′N and 16°03′E) and *Picea abies* (L.) Karst. (Śnieżka Forest District, 50°55'N and 15°46'E; altitude - 400–600 m). Twelve strains of *H. annosum*, four each of the P-, S- and F-IS groups were selected for the study.

The growth conditions of plants and inoculation procedure were described by Werner and Idzikowska (2001) and by Werner and Łakomy (in press).

Analysis of variance (Anova/Manova), Tukey's HSD test, and Student's test were conducted using statistical analysis software Statistica PL 1997 (StatSoft Polska Inc., USA). Data in percentage were transformed before the analysis according to formula of C. I. Bliss of the form: $\arcsin\sqrt{percentage/100}$.

Field inoculation experiments

Three-year-old trees of *P. sylvestris, P. abies* and *A. alba* originated from 'Wanda' forest nursery (Przedborów Forest District, Poland) were used. Trees were planted in the field one year before inoculation. *H. annosum* was represented by twelve strains of the three IS groups.

This part of the study was divided into two experiments: Experiment 1, where narrow wounds (about 10 mm long) were made with a sterile knife, 5 cm above the root collar; Experiment 2, where radial holes (3 mm in diameter) were made with a drill in stems about 5 cm above the root collar. In the both experiments, beech dowels colonized by mycelia of the fungi were inserted into the wounds and protected with Parafilm. Each pathogen × host treatment was replicated ten times. Six months after inoculation, the plants were removed from soil and the extent of necrosis was measured. As controls, ten plants each of pine, spruce and fir were treated with a sterile beech dowels. Two-way analysis of variance, based on individual data, and Tukey's HSD test were conducted.

Greenhouse inoculation experiments

Two experiments were conducted. In Experiment 1, twenty surface sterilized seeds each of Scots pine, Norway spruce and Common fir of this same origin as described above were seeded per container on substrate containing peat and gravel (3:1 v/v). Sawdust overgrown by mycelia of twenty strains of *H. annosum*, four each of the P-, S- and F-IS groups were put in the holes in the substrates close to the roots. In Experiment 2, four-month-old seedlings of the host-plants were planted out on substrate composed of peat, sand and inoculum (3:1:1 v/v). The control plants were grown on uninfested substrates. The plants were randomized on greenhouse benches and the experiments were replicated twice. The plants were harvested two years later in Experiment 1, and 10 months later in Experiment 2. Subsequently, they were divided into roots, dried in the open air and then in the oven at 60°C for 3 days and weighed. The virulence of pathogen was expressed in term of shoot : root ratio (S/R). One-way analysis of variance (Anova) and Tukey's HSD test were conducted.

RESULTS

In vitro, all strains of IS groups were more virulent on spruce than on pine ($p<0,05$) (Fig. 1A). Mean mortality was 53.91% in spruce and 38.43% in pine. Variation in mortality rate among all strains of *H. annosum* and three IS groups was highly statistically significant. The host × strain and host × IS group factors were statistically significant. The components of variance in seedling mortality were specifically attributable to the strain and IS group effects and the interaction factors (Tables 1 and 2).

Table 1. Analysis of variance of mortality for host-intraspecific specialization by twelve strains of *Heterobasidion annosum* representing P, S and F intersterility groups on pine and spruce in pure culture.

Source of variation	**df**	**MS**	**F**	***P***	**% total variation**	**% explained variation**
Host-plant	1	0.404035	58.43	0.0000	11.65	12.24
Strain	11	0.179433	25.95	0.0000	56.94	59.80
Host-plant × strain	11	0.083916	12.14	0.0000	26.63	27.96
Error	24	0.006914			4.78	
Total	47					

Table 2. Analysis of variance of mortality for host-intraspecific specialization by three intersterility groups of *Heterobasidion annosum* on pine and spruce in pure culture.

Source of variation	**df**	**MS**	**F**	***P***	**% total variation**	**% explained variation**
Host-plant	1	0.404035	9.80	0.0032	11.65	23.27
IS group	2	0.413002	10.02	0.0003	23.83	47.57
Host-plant × IS group	2	0.253187	6.14	0.0046	14.61	29.16
Error	42	0.041200			49.91	
Total	47					

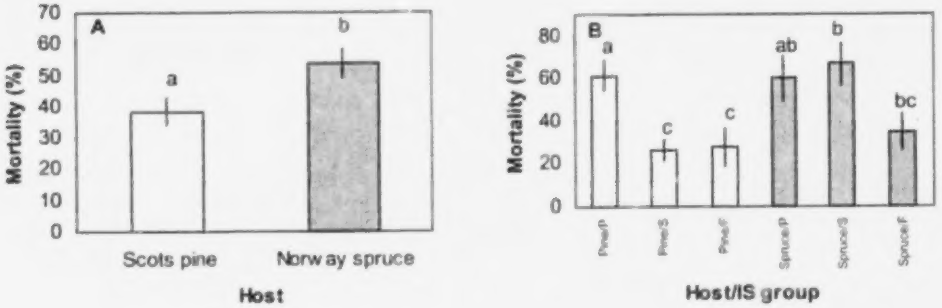

Figure 1. Mean mortality of Scots pine and Norway spruce seedlings inoculated *in vitro* with P, S, and F strains of *Heterobasidion annosum* (A), host preference of the P-, S- and F-IS groups on pine and spruce (B). Bars represent standart error for each group. Means designed by the same letter did not differ significantly at the 5% level using Tukey's HSD test.

All strains of the S group caused significantly higher mortality on spruce seedlings (66.76%) (Fig. 1B) and significant lower mortality ($p<0.0002$) of pine seedlings (Fig. 1B). The P-group isolates displayed similar mortality on both hosts, and the differences were not statistically significant. The F-group isolates were less virulent than P isolates on pine and P and S isolates on spruce; however, they killed significantly more ($p<0.05$) spruce seedlings.

Results of the analyses of variance for vertical spread of *H. annosum* strains and the P-, S-, and F- IS groups in pine, spruce and fir stems are presented in Tables 3, 4, 5 and 6. In both experiments, there were significant differences between hosts ($p<0.05$) and between strains ($p<0.001$). In the interspecific test with three hosts and three IS groups, the differences between host-plants were not statistically significant. There were also no significant differences between IS groups. Higly significant host x IS group factor ($p<0.001$) suggests the existence of host preferency. In the first experiment, the strain and IS group effects accounted for the highest portion of the explained variation. The host-plant accounted for the smallest portion (Tables 3 and 4). Contrarily, in the second experiment, host and IS groups accounted for the similar portions of the explained variance (Tables 5 and 6). In both experiments, the host x strain and the host x IS group factors accounted for the largest portion of variance. Differences in the vertical spread of *H. annosum* mycelium in the sapwood of the three hosts are presented on Figure 2A, B.

Table 3. Analysis of variance for vertical spread of *Heterobasidion annosum* strains representing P, S and F intersterility groups in stems of pine, spruce and fir (Experiment 1).

Source of variation	df	MS	F	P	% total variation	% explained variation
Host-plant	2	1602.01	3.1421	0.0446	1.5115	5.4760
Strain	11	1694.72	3.3239	0.0002	8.7944	31.8608
Host-plant × strain	22	1666.57	3.2687	0.0000	17.2966	62.6632
Error	301	509.85			72.3975	
Total	336					

Table 4. Analysis of variance for vertical spread of *Heterobasidion annosum* strains representing P, S and F intersterility groups in stems of pine, spruce and fir (Experiment 1).

Source of variation	df	MS	F	*P*	% total variation	% explained variation
Host-plant	2	1573.70	2.8822	0.0574	1.4678	8.9069
IS group	2	5212.81	9.5473	0.0000	4.8622	29.5036
Host-plant × IS group	4	5440.93	9.9651	0.0000	10.1499	61.5895
Error	328	545.99			83.5201	
Total	336					

Table 5. Analysis of variance for vertical spread of *Heterobasidion annosum* strains representing P, S and F intersterility groups in stems of pine, spruce and fir (Experiment 2).

Source of variation	df	MS	F	*P*	% total variation	% explained variation
Host-plant	2	104.52	3.338	0.0368	1.7869	7.1266
Strain	11	100.59	3.213	0.0003	9.4584	37.7228
Host-plant × strain	22	73.53	2.349	0.0007	13.8282	55.1051
Error	280	31.30			74.9264	
Total	315					

Table 6. Analysis of variance for vertical spread of *Heterobasidion annosum* strains representing P, S and F intersterility groups in stems of pine, spruce and fir (Experiment 2).

Source of variation	df	MS	F	*P*	% total variation	% explained variation
Host-plant	2	91.9831	2.6187	0.0745	1.5515	17.1338
IS group	2	91.6581	2.6094	0.0752	1.5460	17.0732
Host-plant × IS group	4	173.6057	4.9424	0.0007	5.9578	67.7930
Error	307	35.1252			90.9446	
Total	315					

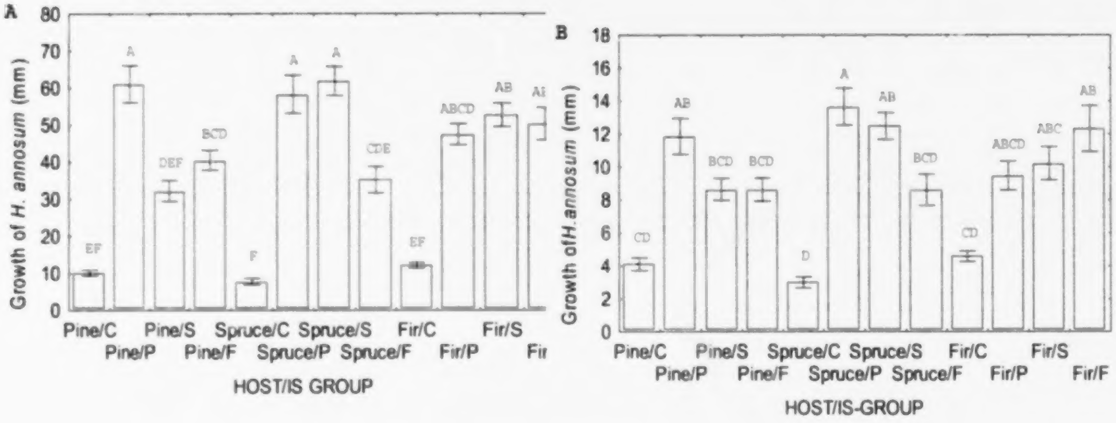

Figure 2. Growth of P, S and F isolates of *Heterobasidion annosum* in pine, spruce and fir stems in Experiment 1 (A) and Experiment 2 (B) in comparison with control. Bars represent standart error for each group. Means designed by the same letter did not differ significantly at the 5% level using Tukey's HSD test.

Quite different biomasses of pine, spruce and fir seedlings of the same age in the greenhouse experiments inable to use two-way analysis of variance and in a consequence to speculate about the host preference. In the intraspecific analysis with one host and three IS groups, the statistically significant differences for shoot : root ratio were observed on pine in the Experiment 1 and in the Experiment 2 on spruce and fir (Fig. 3). The results of the analysis of variance for the effects of strain and IS group on shoot and root biomasses, and shoot : root ratio are shown in table 7.

Table 7. Analysis of variance probabilities (p<F) of main effects of strain and IS group on dry weight of shoots, roots and shoot : root ratio of Scots pine, Norway spruce and Common fir inoculated with twelve strains of *Heterobasidion annosum* representing P-, S-, and F-intersterility groups under greenhouse conditions.

Traits	Scots pine		Norway spruce		Common fir	
	Strain	IS group	Strain	IS group	Strain	IS group
Dry weight of shoots						
Experiment I	<0.0001	0.018	<0.0001	<0.0001	<0.0001	0.0041
Experiment II	NS	0.0085	<0.0001	NS	0.0002	NS
Dry weight of roots						
Experiment I	<0.0001	NS	<0.0001	<0.0001	<0.0001	NS
Experiment II	0.002	0.0037	0.0208	NS	NS	NS
Shoot : root ratio						
Experiment I	<0.0001	<0.0001	0.009	NS	NS	NS
Experiment II	NS	NS	0.0352	NS	<0.0001	0.0238

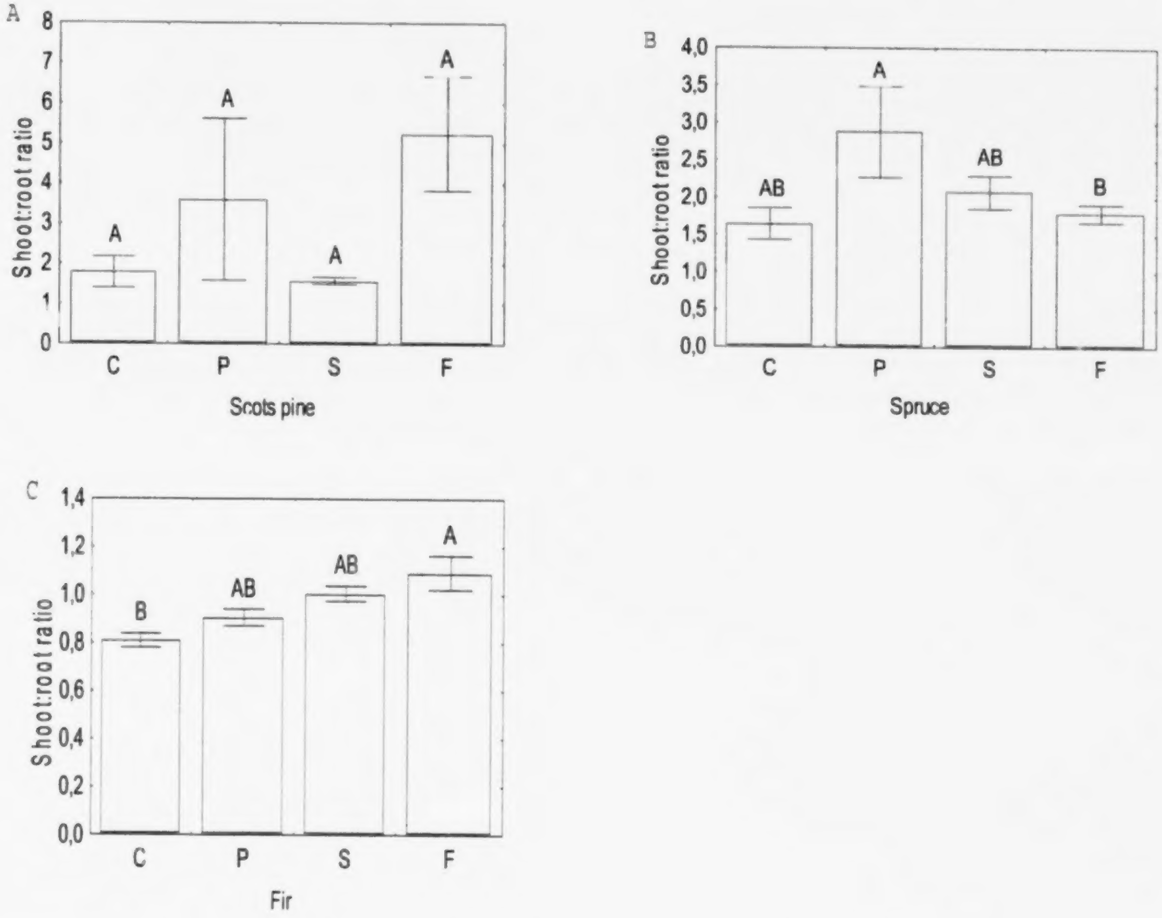

Figure 3. Shoot : root ratio of pine, spruce and fir seedlings inoculated with P, S and F strains of *Heterobasidion annosun* in greenhouse Experiment 1. Bars represent standart error for each group. Means designed by the same letter did not differ significantly at the 5% level using Tukey's HSD test.

DISCUSSION

The results of the three different inoculation experiments provide strong evidence for the occurrence of the intraspecific variation in *H. annosum* complex similar to that observed in nature, which is interpreted as an early step towards the species differentiation (Korhonen 1978). The results of the inoculation experiment *in vitro*, suggest that using seedlings grown *in vitro* to test virulence and host preference is an alternative method to measurement of fungal spread in sapwood of older trees. Moreover, contrary to other artificial infection experiments, the final outcome of host-pathogen interactions was largely dependent on host and pathogen genotypes, these results can contribute to better understanding the nature of host preference. In this experiment, the isolates and IS groups accounted for most of the explained variation in the host mortality. Portion of explained variation due to the effect of host-plant was far more smaller. In this respect, the results are similar to those obtained in the field experiments. Inability to use two-way analysis of variance for data of our greenhouse inoculation experiments makes impossible to draw a comparison with the results of the experiments described above. Despite lack of statistically significant differences between the effect of IS groups on shoot : root ratio in the greenhouse experiments, virulence of the IS-group strains (particularly in the second experiment, where roots of plants were probably more injured because of replanting) showed similar tendency to that observed in *in vitro* and field inoculation experiments.

The results of another greenhouse experiments, in which wounding methods were used, provided similar, strong evidence for host preferency. In the study by Kuhlman (1970), variation in host susceptibility due to host, isolate, and host × isolate interaction was significant at the 1% level. A significant interaction between pine and Douglas-fir isolates (ascribed later to the North American P- and S-IS groups by Harrington et al. 1989) and *Pinus ponderosa* and *Abies concolor* was showed by Worrall et al. (1983). In greenhouse inoculation experiments on 4-year-old trees of Scots pine and Norway spruce (Stenlid and Swedjemark 1988), S- group isolates easily infected spruce but showed limited growth on pine, whereas P isolates aggressively attacked both hosts. Spruce was more susceptible than pine. Also, in the study by Swedjemark et al. (1999), the P-group isolates were more virulent on pine than the S-group isolates. In spruce, P isolates were also more virulent, but the differences between isolates of the both IS groups were not statistically significant. Similarly, in our inoculation experiment, the host preference was the most distinct among S isolates to spruce, whereas there was almost no difference in mortality rate and longevity of necrosis in stems between S and P strains on spruce. The F-group izolates were the most virulent on fir, albeit the difference was not statistically significant. This may reflect their higher saprobic properties (Capretti et al. 1990; Łakomy et al. 2000).

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BUTT ROT OF OLD GROWTH OF *CHAMAECYPARIS PISIFERA* CAUSED BY *SERPULA HIMANTIOIDES*

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SUMMARY

Incidence of butt rots and the pathogens were studied in a clear-cut area in a 131-year-old plantation of *Chamaecyparis pisifera* (Sawara) and *Cryptomeria japonica* (Sugi) in Mito City, Japan. Decay was found in 91% of 122 Sawara and 42% of 84 Sugi stumps. Decay was serious in Sawara trees and the decayed part exceeded 30 cm in diameter on the cut surface in 22% of Sawara stumps. Thirteen basidiomycetous cultures were isolated from brown-rotted wood of Sawara stumps and three basidiomycetous cultures from white-rotted wood of Sawara or Sugi stumps. The brown-rot cultures showed similar cultural characteristics on agar media and appeared to be one single species. Basidiocarps of wood-decay fungi were collected in the study site and cultures were isolated. One of the collected basidiocarps was identified to be *Serpula himantioides*. The cultures isolated from *S. himantioides* showed similar cultural characteristics as those of the brown-rot cultures. The brown-rot fungus was proved to be *S. himantioides* by a mating experiment that was done between a monosporous culture of *S. himantioides* and monokaryotized cultures of the brown-rot fungus treated with 0.25% oxgall. *Serpula himantioides* is first reported to have caused decay in living trees from Japan.

Keywords: brown rot, butt rot, *Chamaecyparis pisifera*, Japan, *Serpula himantioides*

INTRODUCTION

A small forest is reserved in the central part of Mito City, Ibaraki prefecture, Japan. Most part of the forest is covered with old growth of *Cryptomeria japonica* D. Don (Sugi) and *Chamaecyparis pisifera* Endl. (Sawara). Wind fall occasionally occurs in the forest due to strong wind, especially typhoon. As the forest is located in a residential district and surrounded by houses and public institutions, the forest management office decided to cut trees in the margin of the forest to avoid any accident caused by wind fall. When these trees were cut down, serious decay damages were found in many trees, especially in Sawara. The authors made a field survey on decay damages in the clear-cut area of the forest and tried to identify the causal fungi by isolation of cultures from decayed stumps.

MATERIALS AND METHODS

The study site is called as Fudoyama, located in the Kasahara town in Mito City, Ibaraki prefecture. Total area of the forest is 6.3 ha, the land shape is almost flat and at 30 m above sea level. The forest is a plantation mostly consisting of 131-year-old Sawara and Sugi. The north and west margins of the forest were clear-cut approximately 30 m wide in August of 1999. Decayed stumps were examined in the clear-cut site in October and November of 1999 and in May and June of 2000. Tree species, stump diameter, decay size and type of decay were recorded for each stump and a location of a stump was plotted on a map. Stump diameter and decay size were measured on the cut surface of each stump. Tree species of stumps were identified by wood appearance and microscopic observation. Cultures were isolated from decayed stumps by picking up small pieces of decayed wood, placing them on potato dextrose agar (PDA) plate and incubating at 25°C.

Basidiocarps occurring on decayed timbers or stumps were collected in the forest once every two weeks from May to November in 1999 and 2000. To confirm a causal fungus of the decay, a mating experiment was done between the cultures isolated from the collected basidiocarp and decayed stumps. Cultures were isolated from many basidiospores or a single basidiospore of the basidiocarps. Monokaryotic cultures were produced by chemical monokaryotization. Dikaryotic cultures from decayed wood were cultured on PDA at 25°C containing 0.1, 0.2, 0.3, 0.4, 0.5 or 0.6% sodium cholate or 0.1, 0.25, 0.5 1.0 1.5 or 2% oxgall (Takemaru 1964). Six cultures (WD-2168 2169 2172 2174 2175 and 2176) isolated from decayed wood were treated with the chemicals. The mating experiment was done between single-spore culture (WD-2186) from the basidiocarp and the monokaryotized cultures from the decayed wood. Small pieces of agar mycelia of two cultures were placed 2 cm apart each other on PDA plate and incubated at 25°C. After both mycelia overlapped on agar, formation of clamp connections were checked under a microscope.

A wood decay experiment was carried out to confirm decaying abilities of the cultures. Small wood blocks (1.5 x 1.5 x 2 cm) were placed in the sand medium (60 g of perlite mixed with 60ml of 2% malt extract) in a plastic bottle, autoclaved, inoculated with the cultures and incubated at 25°C for 3 months. Three cultures (WD-2169 2174 2178) from the decayed wood and two cultures (WD-2183 2184) from the basidiocarp were used for the decay experiment. Average weight loss was obtained by measuring dry weight of five wood blocks before and after the experiment for each bottle.

RESULTS AND DISCUSSION

The clear-cut area is L-shaped, ca. 30 m wide 195 m long from east to west and 187 m long from north to south. One hundred and twenty two stumps of Sawara and 84 stumps of Sugi were examined. The locations of stumps in the clear-cut area were shown in Fig. 1. Sawara stumps were mainly distributed in northern margin of the forest and Sugi stumps were mostly in southern margin, but some Sugi stumps were in northern margin. Average diameter of stumps at the cut surface were 92.4 cm in Sawara and 80.0 cm in Sugi. Ninety one percent of Sawara and 42% of Sugi were decayed.

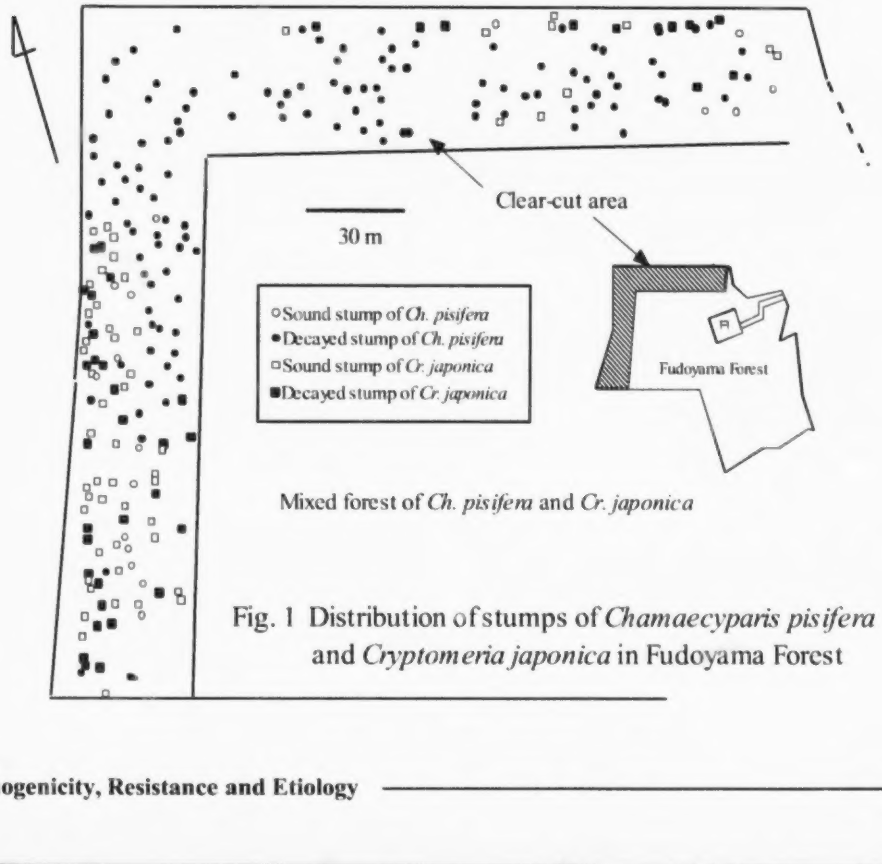
Clear-cut area
30 m
○Sound stump of *Ch. pisifera*
●Decayed stump of *Ch. pisifera*
□Sound stump of *Cr. japonica*
■Decayed stump of *Cr. japonica*
Clear-cut area
Fudoyama Forest
Mixed forest of *Ch. pisifera* and *Cr. japonica*

Fig. 1 Distribution of stumps of *Chamaecyparis pisifera* and *Cryptomeria japonica* in Fudoyama Forest

The diameter of the decayed part exceeded 10 cm at the cut surface in 84% of Sawara stumps, but in only 10% of Sugi stumps. Serious decays, exceeding 30 cm in diameter, were found in 22% of Sawara stumps, but in only 2% of Sugi stumps (Table 1). The decay damages were much serious in Sawara than Sugi trees. It is not clear that the phenomenon is due to the durability of wood in each tree species or other reasons.

Table 1. Number and percentage of decayed stumps of *Chamaecyparis pisifera* and *Cryptomeria japonica* in Fudoyama forest.

Diam. of decayed area of cut surface of stumps	*Ch. Pisifera*		*Cr. japonica*	
	No. of stumps	%	No. of stumps	%
0	11	9	35	58
< 10 cm	8	7	25	30
10-20 cm	53	43	7	8
20-30 cm	23	19	1	1
30 cm <	27	22	2	2
Total	122	100	84	100

Brown rot was found in 75 stumps (68% of decayed stumps) and white rot in 20 stumps (18%) in Sawara (Table 2). In Sugi, brown rot was found in 14 stumps (40% of decayed stumps) and white rot in five stumps (14%, Table 3). Heart rot was found in 104 stumps (94% of decayed stumps) and sap rot in 43 stumps (39%) in Sawara. In Sugi, heart rot was observed in 32 stumps (91% of decayed stumps) and sap rot was observed in three stumps (9%). The damages of brown rot appear to be much larger than those of white rot and heart rot was more frequently found than sap rot.

Table 2. Type of rot in the decayed stumps of *Chamaecyparis pisifera*.

Type of rot	Heart rot	Sap rot	Heart + sap rot	Total
White rot	8	1	6	15
Brown rot	47	2	21	70
W + B rot	3	0	2	5
Unknown	10	4	7	21
Total	68	7	36	111

Table 3. Type of rot in the decayed stumps of *Cryptomeria japonica*.

Type of rot	Heart rot	Sap rot	Heart + sap rot	Total
White rot	0	0	5	5
Brown rot	4	1	9	14
W + B rot	0	0	0	0
Unknown	3	2	11	16
Total	7	3	25	35

Isolation was done from decayed wood of stumps and 13 cultures were isolated from brown-rot part of Sawara stumps. One culture was isolated from white-rot wood of Sugi stump and two cultures from white-rot wood of Sawara stamps. Thirteen cultures, isolated from decayed wood of Sawara, showed the same cultural characteristics: mat cottony, white at first, later becoming brownish orange to light brown, producing mycelial cord on medium; hyphae hyaline to pale brown, with single to multiple clamp connections. They appear to be one single species of wood-decay fungi. The culture isolated from Sugi stump, having clamp connections on hyphae, producing white mycelia and coremia with black conidiospores on PDA, were identified to be *Pleurotus cystidiosus* O.K. Miller. Two white-rot cultures remain to be unidentified.

For identifying the brown-rot isolates, basidiocarps of wood-inhabiting fungi were collected from the uncut area of the forest. *Fomitopsis carnea* (Bl. et Nees) Imaz., *Gloeocystidiellum* sp. and *Serpula himantioides* (Fr.: Fr.) Karst. were collected and cultures were isolated from them. Among them, the isolates of *Serpula himantioides* closely resembled to the brown-rot fungus in macro- and micromorphology. Three cultures were isolated from multi-basidiospores of *S. himantioides* (WD-2184 2185 2186) and one culture from a single basidiospore (WD-2186).

By chemical treatment, monokaryotization occurred in the isolates grown on PDA containing 0.25% oxgall after 2-months incubation, but there was no change in the cultures treated with sodium cholate and oxgall of the other concentrations. All the six isolates tested were monokaryotized and only simple-septa were observed in their colonies. Monokaryotized isolates were stable and no morphological change was observed for 6 months after monokaryotization.

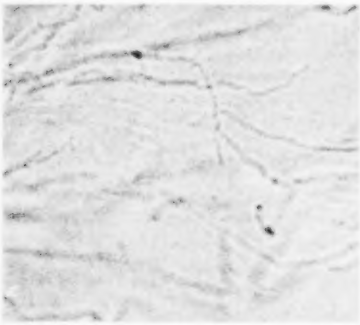

Fig. 2 Monokaryotized hyphae (WD-2172m)

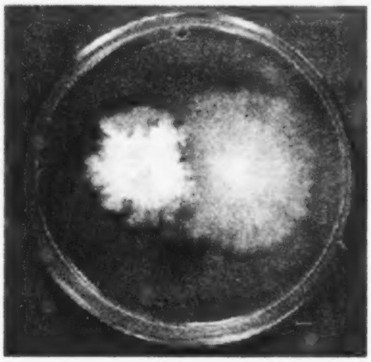

Fig. 3 Mating experiment on PDA WD-2186 (left), WD-2176m (right)

Six monokaryotized isolates were mated with the single-spore isolate of *S. himantioides* and clamp connections were observed on the hyphae in the overlapping parts of two cultures within two weeks in the all combinations. The mating experiments shows that the brown-rot isolates from decayed wood are identical with those from basidiospores of the basidiocarpe, that is *S. himantioides*.

In the decay experiment, all the cultures caused significant weight (ca. 15-30%) losses during 3-months incubation (Table 4). WD-2184, isolated from basidiospores of *S. himantioides*, caused the greatest weight losses in the cultures tested. There were no significant weight changes in control, but there might be some in tolerance. Harmsen (1978) reported that decay rate of *S. himantioides* is very rapid, causing 50-60% weight loss in sapwood of *Pinus sylvestris* after 12 weeks in soil-block culture. The fungus has great ability for decaying softwood. As wood blocks of Sawara were not available for the present study, durability of Sawara wood remained unknown. Further decay experiments should be done using wood blocks of Sawara.

Strain no.	***Cr. Japonica***		***Ch. obtusa***
	Sapwood	**Heartwood**	**Sapwood**
WD-2169[a]	20.0	18.8	18.0
WD-2174[a]	24.0	21.7	20.6
WD-2178[a]	18.1	17.6	14.8
WD-2183[b]	21.0	22.8	18.3
WD-2184[b]	29.6	29.9	28.7
Control	0.7	+0.2	+0.2

[a] Isolated from decayed wood

[b] Isolated from basidiospores

Serpula himantioides have merulioid hymenium, brown basidiospores and dimitic hyphal system. The fungus resembles *S. lacrymans* (Wulf.: Fr.) Schroeter, but the former has thinner and resupinate basidiocarps (Hallenberg and Eriksson 1985). *Serpula himantioides* often causes decay of dead wood and timbers, but was also reported to cause butt rot of living conifers and hardwoods in Europe (Harmsen 1978; Siepmann 1984; Seehann 1986), Russia (Aref'ev 1991) and North America (Whitney 1995). However, the fungus has been known to be a saprophyte of dead wood and commercial timbers in Japan. This is the first report on *S. himantioides* causing decay of living trees from Japan.

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CHARACTERIZATION OF FUNGAL ISOLATES FROM *NEWTONIA BUCHANANII* TREES IN SUB–MONTANE RAIN FOREST IN TANZANIA

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ABSTRACT

Of 23 fungal strains isolated from standing *Newtonia buchananii* trees, 30% showed a positive Bavendamn reaction, which suggests their ability to produce phenol oxidase and to deplete polysaccharide and lignin components in wood. Comparative pathogenicity tests using *N. buchananii* as a host and Scots pine as non-host seedlings were conducted by inoculation with three of the isolates (*Amphisphaeria spp.* code AF437752, *Hypocrea rufa* code AF437749 and *Chaetomium* spp. code AF437778. The extent of fungal growth millimetre (mm) and percentage number of cell deaths were used as indices to evaluate extent of host responses. The experiment was also repeated using known pathogenic fungi of conifer trees *Heterobasidium annosum* and an obligate saprotroph (*Trichoderma auroviride*). Interestingly, *H. rufa* code AF437749 caused 34% cell death in Scots pine as well as 3 mm and 23 mm decay column in *Newtonia buchananii* seedlings, radial and axial respectively. However, this cell death (34%) was comparatively less compared to *H. annosum* in Scots pine. Notably, the other fungal strain (*Chaetomium* spp. code AF437778) also caused decays in *N. buchananii* and significant cell death as much as *H. annosum* on Scot pine. As expected, inoculation with *T. auroviride* did not provoke a strong necrotic cell-death-related host response. *Amphisphaeria spp.* and *Chaetomium spp.* also damaged epidermal cells and Casparian band strips, which indicates that apoplast substance pathway into stele, may have occurred. These results, therefore, suggest the involvement of these three isolates (*Chaetomium* spp. *H. rufa* and *Amphisphaeria spp* in decay of *N. buchananii* trees in rainforests. However, further studies may be needed to establish the pathogenic capability of the isolates under field conditions.

Keywords: Pathogenicity test, cell death, endophytes, ascomycetes, butt rot, *Newtonia buchananii*

INTRODUCTION

Root and butt rot pathogens appear to be biotic disturbance agents, which are found in the tropical montane rainforests. A good number of studies shows that tropical rainforest trees suffer from a range of fungal pathogens (Renvall and Niemelä 1993, Nsolomo and Venn 1994, Gilbert et al. 1994, Gilbert 1995, 1996). However, pathogenic species diversity and their role in the ecosystems are not clearly covered. For example, in Tanzania, the canopy tree species growing in the montane rainforests suffer from root and butt rot diseases (Renvall and Niemelä 1993, Nsolomo and Venn 1996). Together with other small-scale disturbance agents, these pathogens cause wood decay and tree fall, thus creating large canopy gaps. Most documented species infected by root and butt rot pathogens are the emergent species such as *Ocotea usambarensis* (East African camphor), and *Newtonia buchananii* (Baker) Gilb & Bout (Leguminosae, Mimosoideae). Renvall and Niemelä (1993) reported 10 polypore species (*Basidiomycetes*) all causing wood decay in *O. usambarensis* growing along the West Usambara Mountains. Frequently isolated pathogens infecting canopy trees in Tanzania include *Phellinus senex* (syn. *Fomes senex; Polyporus*) and *Loweporus inflexibilis* (Renvall and Niemelä 1993, Nsolomo and Venn 1994). However, fungal pathogens attacking *N. buchananii* trees have not been well covered.

N. buchananii is one of emergent high canopy tree species, one widely scattered in submontane rainforest in Tanzania. It produces valuable timber suitable for joinery in building and constructions work (Bryce 1967).

Nevertheless, butt rot of living *N. buchananii* seems to be a serious problem on trees growing in Mazumbai Forest Reserve and in its surrounding human disturbed forests (Mrema and Nummelin 1998). *Newtonia* trees severely infected by root and butt rot, fall and form open canopy gaps in the reserve. As noted by Struhsaker et al. (1989) in Kibale forest, Uganda, severe dieback is another reported disease attacking *Newtonia* trees. With the exception of these two cases (butt rot and dieback disease), not much has been documented on biotic disturbance agents affecting *N. buchananii* trees, and their mode of attack.

The objective of this study was to characterise fungal isolates from *N. buchananii* trees with the intention of isolating those pathogenic fungi causing decay in living *Newtonia* trees. Categorisation of the isolates was performed according to their ability to produce extra-cellular oxidase enzyme. Necrosis reaction of non host and host species challenged by fungal strains positive in Bavendamn reaction was evaluated, to characterise and elucidate the ability of those phytopathogenic fungi to cause disease on living tissues.

MATERIALS AND METHODS

Fungal strains

All tested fungal strains, (except *Chaetomium* spp. code AF437778), were isolated from small *N. buchananii* trees with diameter range of 30 - 60 cm. *Chaetomium* spp (code AF437778) was isolated from *N. buchananii* seeds as a seed-borne fungus. An increment borer (4 mm) diameter was used to extract the cores from stem wood of standing trees at breast height (1.3 m); cores were kept in plastic bags to protect them from external contamination before they were transported to the laboratory. In the laboratory, each core was surface-sterilised by light flaming withan alcohol burner. It was then cut and separated into four categorises: (A), as healthy sapwood; (B), as incipient discoloured wood; (C) as discoloured wood; and (D) as highly discoloured decayed wood. Each specimen in each category was further cut into three portions and cultured on a 90 mm petri dish containing growth medium (2% malt extract agar). Isolation of fungal strains was conducted on each second specimen among the three placed on each petri dish. Characteristics of fungal strains used in this experiment are as shown in Table 1. For comparison, *Heterobasidion annosum* and *Trichoderma auroviride* from known origin were included in this study.

Table 1. Fungal characteristics.

Isolates	Genebank accession number for ITS sequences	Characteristics	Source
**Amphisphaeria spp.*	AF437752	Endophyte & (Pathogen)	(Worapong et al. 2001) (Gilbert and Hubbell 1995)
**Hypocrea rufa*	AF437749	Biological- control	(Lieckfeldt et al. 1999)
**Chaetomium, spp*	A43777	Seed borne & Softrot fungus	(Mamta et al. 1996) (Kamdem and Mcintyre 1999)
Heterobasidion annosum	FP5 P-type	Necrotroph	(Asiegbu et al. 1999)
Trichoderma auroviride	A361	Saprotroph	(Asiegbu et al. 1999)

* Tested fungal strains

Characterisation of fungal strains

Except for the media used to test total cellulase, in which glucose was not needed, all fungal isolates used in this study had been tested for enzyme production (cellulolytic and lignolytic), using nutrient solution containing (g/l); NH_4NO_3 0.6 g, K_2HPO_4 0.4 g, $KHPO_4$ 0.5 g, $MgSO_4.7H_2O$ 0.4 g, and glucose 5.0 g. In the Bavendamn reaction test, the medium solution contained 0.1% guaicol at pH 5.5 and 2.5% agar and assessment was done seven days after inoculation with tested strains. In a medium solution used for total cellulase, 0.1% cellulose powder was added. Congo red was flooded on agar plates inoculated with fungal strain, 5 days after inoculation. Ligninase activity was estimated 7 to 14 days after inoculation of tested strain on medium containing 0.1mg/ml of remazol brilliant blue dye. The extent of polymeric dye discolourization was calculated to estimate total enzyme production in percentage. The selection of fungal strain was based on the extent of enzyme activity production.

Plant material and inoculation with fungal strain

Non host

Scots pine (*Pinus sylvestris* L.) seeds were surface-sterilised with 33% H_2O_2 for 10 minutes under agitation on a shaker and then rinsed several times with sterile water. The seeds were then sown on sterile 1% water agar medium and germinated at 22°C in a growth chamber in darkness. Scots pine seedlings (14 days) were transferred into a second set of Petri dishes with 1% water agar and overlaid with half moist sterile filter paper prior to transfer of seedlings as described in (Asiegbu et al. 1993, 1999). Then 1ml of mycelium suspension was applied and a second half-moist filter paper was used to cover the seedling roots. The root region half of each plate was covered with aluminium foil and kept under a photoperiod of 16 h light at 20°C (200 □Em^{-2}s^{-1} of PAR) in according to (Asiegbu et al. 1993, 1999).

Host plant

Two-year-old seedlings of *N. buchananii* grown in the greenhouse at the Swedish University of Agricultural Sciences were used as host species in this study. All seedlings were irrigated daily and fertilised with complete mineral nutrient (Walco 100 g/l) every week. The stems were surface-sterilised using 70% alcohol, then wounded to the xylem and applied tested fungal innoculum (*Amphisphaeria* spp, *Chaetomium*, spp, *H. rufa*) previous grown on sterilised *N. buchananii* saw dust. Control plants were mock inoculated with sterile 1% w/v water agar, then covered with parafilm to prevent external contamination.

Cell death determination under fluorescence microscopy

Non suberized seedling roots of Scots pine were used in this study as non host control plants Percentage cell death rate in the first and second 10-mm root region was evaluated at 3, 5, 7 and 12 days post inoculation (p.i). Samples (five root tips) were first hydrolysed in 3% HCL in ethanol for 5 minutes at room temperature and then washed in phosphate-citrate buffer pH 3.8. Staining was performed in 0.001% acridine orange for 15 minutes and rinsed twice in phosphate-citrate buffer, pH 3.8, for 5 minutes each. Sample examination was conducted under a Leitz orthoplan fluorescence microscope, with exciting filters BG12 and BG8 and suppression filter K510, by counting the greenish yellow fluorescing nuclei within the microscope field of view using a X40 objective field (Kacprzak et al. 2001). Three fields of view per root in the first 10-mm root tips and then 20-mm root tips were examined.

Light microscopy and Transmission electron microscopy (TEM) tissue preparations

To study the pre-infection process, the first 10-mm root tips were cut from the inoculated and control (un-inoculated) seedlings 3, 6, 10 and 15 days post inoculation. Study material was fixed in 3% v/v glutaraldehyde in 0.2M phosphate buffer (pH 7.2) for 3 to 4 h and thereafter washed in three consecutive steps in 0.1M buffer and rinsed two successive steps in distilled water. The specimens were dehydrated in an ethanol series and infiltrated using ethanol and London resin (LR) series. Finally, they were embedded in LR in plastic capsules. For light microscopy, □□m thin section were obtained and stained with toluidine blue. For TEM, 70 nm thin sections were made and collected on nickel grids and observed under transmission electron microscope before photos were taken.

Scanning electron microscopy (SEM)

Material for SEM (2-3 root tips) was post-fixed in 0.2% w/v aqueous osmium tetroxide for 1 h, followed by three successive washing steps in 0.1M phosphate buffer and rinsed twice in distilled water. The specimen was dehydrated in an ethanol series (Asiegbu et al. 1993) and further dehydrated in an ascending acetone ethanol series (1:3, 2:2, 3:1), and pure acetone for 30 min, 30 min, 1 hr and 12 hr, respectively). All specimens were coated and examined with a scanning electron microscope.

RESULTS

Characterisation of fungal strains

Of all fungal strains isolated from *N. buchananii*, only 30% showed a positive Bavendamn reaction (Plant responses) to the fungal disease. Table 2 shows the percentage of enzyme activity tests for fungal strains used in this study. *Chaetomium spp.*, *Amphisphaeria spp.* and *H. annosum* showed strong enzyme reaction (38.0, 33.6 and 39.9% respectively). *H. rufa* and *T. auroviride* showed a weak Bavendamn reaction (11.1 and 9.9%), and *H. rufa* showed a very strong cellulase enzyme reaction (49.0%).

Table 2. Enzyme activity by different fungal strains.

Fungal strain	**Bavendamn**		**Cellulase**		**Lignolitic**	
	% activity	**Score**	**% activity**	**Score**	**% activity**	**Score**
Chaetomium spp	38.0	++++	44.7	++++	-Ve	-Ve
Amphisphaeria spp	33.6	++++	-Ve	-Ve	40.3	++++
Hypocrea rufa	11.1	++	49.0	++++	-Ve	-Ve
Heterobasidion annosum	39.9	++++	Nd		Nd	
Trichoderma auroviride	9.9	+	Nd		Nd	

Note: ++++ (over 30 mm) = Very strong; +++ (21 – 30 mm) = Strong; ++ (11 – 20 mm) = Weak; + (1 – 10 mm) = Very weak; -Ve = No reaction. Nd = Not determined.

Cell death responses on Scot pine seedlings (non-host) and *N. buchananii*

Response on pine seedlings challenged with the fungal strains (*Amphisphaeria* spp, *Chaetomium*, spp, *H. rufa*, *H. annosum* and *T. auroviride*) was noted from 3 days after inoculation, but differed in reduction of living cell within the first and second 10 mm root regions, as well as between fungal strain (Figure 1). Figure 1A and 1B, shows that at 5 days after inoculation (Pi), seedlings challenged with *H. annosum*, *Chaetomium* spp and *Amphisphaeria* spp showed a higher percentage of cell death and browning, when compared with those in control seedlings ($P<0.001$ for all). Seedlings inoculated with *H. rufa* and *T auroviride* showed slightly less response (*ca.* 30%) in number of reduced living cells within both regions (first and second 10 mm) by 5 days post-inoculation. Pine seedlings challenged by *H. annosum* and *Chaetomium* spp. had almost 100% cell death, while those inoculated with *Amphisphaeria* spp. showed 85% cell death. Seven days after inoculation, the number of cell deaths within the second 10 mm root region challenged by *H. annosum*, *Chaetomium* spp and *Amphisphaeria* spp was higher (18.7, 7.5, and 28.6%, respectively) than those in the first 10 mm root regions. However, 12 days post-inoculation, the cell death was almost 100% in pine seedlings challenged by *H. annosum* and *Chaetomium* spp., and 85% in those challenged by *Amphisphaeria* spp. *N. buchananii* seedlings were challenged by *Chaetomium spp.*, *Amphisphaeria* spp and *H. rufa*. Strong necrotic browning was noted only in *Chaetomium spp.* and *H. rufa*.

The radial and axial measurement of browning tissue in *Newtonia* caused by *H. rufa* was 3 mm and 23 mm, respectively. Since there was no obvious discoloration compared with control plants, the response by the *newtonia* seedling infected by *Amphisphaeria* spp was not visible to the naked eye.

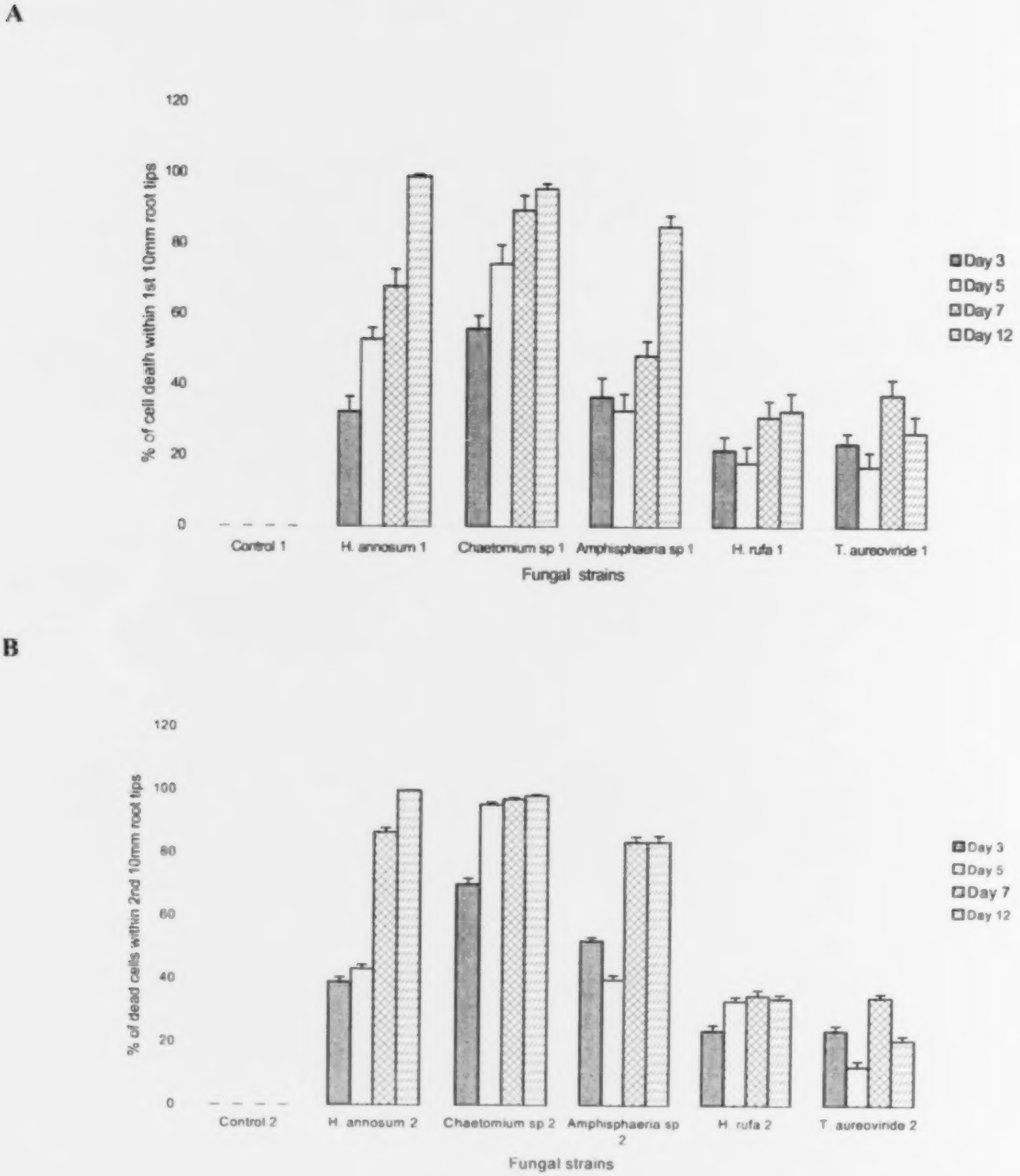

Figure 1. Cell death in Scots pine seedlings challenged by pathogenic and non-pathogenic fungal strains at different days (A: first 10 mm, B: second 10 mm).

Fungal colonisation

Studies on sections from pine roots inoculated by *Chaetomium spp* and *Amphisphaeria* spp showed hyphae invaded in the epidermal and cortical cells within 3 days post-inoculation. Within 5 to 10 days. the hypha were noted between cell walls and in the cells. On day 7, *Chaetomium spp.* hyphae had penetrated into the vascular tissues. At SEM level, penetration by *H. rufa* conidia in Scots pine living tissues was noted in 10 to 15 days post-inoculation (Figure 2). *Newtonia* seedlings challenged by *H. rufa* showed that the ray cells were highly damaged and even closer vessels to ray cells were also invaded. Invasion by *Amphisphaeria* spp. was also noted in xylem fibers and in ray parenchyma cells. Figure 3 shows intra-cellular colonisation and degradation of host tissues by the different fungi isolates tested. Studies on sections from pine roots inoculated by *Chaetomium spp* and *Amphisphaeria* spp showed hyphae invaded in the epidermal and cortical cells within 3 days post-inoculation. Within 5 to 10 days the hypha were noted between cell walls and in the cells. On day 7, *Chaetomium spp.* hyphae had penetrated into the vascular tissues. At SEM level, penetration by *H. rufa* conidia in Scots pine living tissues was noted in 10 to 15 days post-inoculation (Figure 2). *Newtonia* seedlings challenged by *H. rufa* showed that the ray cells were highly damaged and even closer vessels to ray cells were also invaded. Invasion by *Amphisphaeria* spp. was also noted in xylem fibers and in ray parenchyma cells. Figure 3 shows intra-cellular colonisation and degradation of host tissues by the different fungi isolates tested.

DISCUSSION

Ability of fungi to cause disease on living tissues

Results in this study show that the tested fungal isolates differed in extracellular enzyme production, suggesting their differences in depletion of polysaccharide and lignin components in wood. (Table 2). The Bavendamn reaction in *Chaetomium spp.* and *H. annosum* seems to be similar, while that of *Amphisphaeria* spp. was less by 4 to 6%. In order to parasitize a plant successfully, fungi have pathogenicity factors that allow them to overcome the basic resistance of the plants (Heath 1991). On day 12 post-inoculation, the percentage cell death and browning in *Chaetomium spp* and *H. annosum* was significantly higher in both root regions (1st and 2nd 10 mm region). This indicates that hypersensitivity reaction by the seedlings in response to *hrp* gene products from both fungal strains could not stop their infection (Heath 2000). According to Kamdem and Mcintyre (1999), some of *Chaetomium* spp. are suggested to be soft-rotting fungus. In this study, *Chaetomium* spp. was isolated from *Newtonia* seeds as a seedborne fungus, supporting similar studies by (Mamta et al.1996) in some tropical tree species. This suggests that biotic stresses in *Newtonia* trees may originate during very early period of growth at seedling stage.

Amphisphaeria spp. is a perfect stage (telemorph) of *Pestalotiopsis* that has been reported as a taxol-producing fungus and as an endophyte fungus (Strobel et al. 2000) and also as a pathogen (Gilbert and Hubbell 1995). In this study, the tested fungi caused cell death in pine seedling as well as in *Newtonia* seedlings (Figure 2 and 3). This may suggest that this fungus could have a latent stage in their host, and become pathogenic during certain periods, probably due to changes in environmental conditions. The infection of these fungal strains was conducted by wounding the seedlings into xylem, and may have altered the physiological performance of the seedling. However, all seedlings (including the controls) were treated in the same manner. *H. rufa* caused cell mortality up to 34% in pine seedlings, and discoloration in *Newtonia* seedlings. However, the cell death caused by this fungus in pine seedlings was less when compared to *H. annosum*, and this difference was significant at $P < 0.001$. The browning discoloration noted in *Newtonia* seedlings could indicate that necrotic cell death was intended to halt further invasion (Asiebgu et al. 1999). However, the observed hypersensitive (HR.) cell death could not stop most fungal strains used in this study, indicating that *Newtonia buchananii* probably is weak in the compartmentalisation process (Shigo 1983, Shortle 1984). This invasion caused by *H. rufa* could probably be explained by the fact that inoculation was done by wounding to the xylem, and that could have influenced changes in cell biochemistry and physiology (Kobayashi et al. 1995). *Trichoderma viride* is reported to be an anamorph of *H. rufa*. In this study, the percentage of cell death caused by *H. rufa* by day 12 post-inoculation was not significantly different from that of *T. auroviride*.

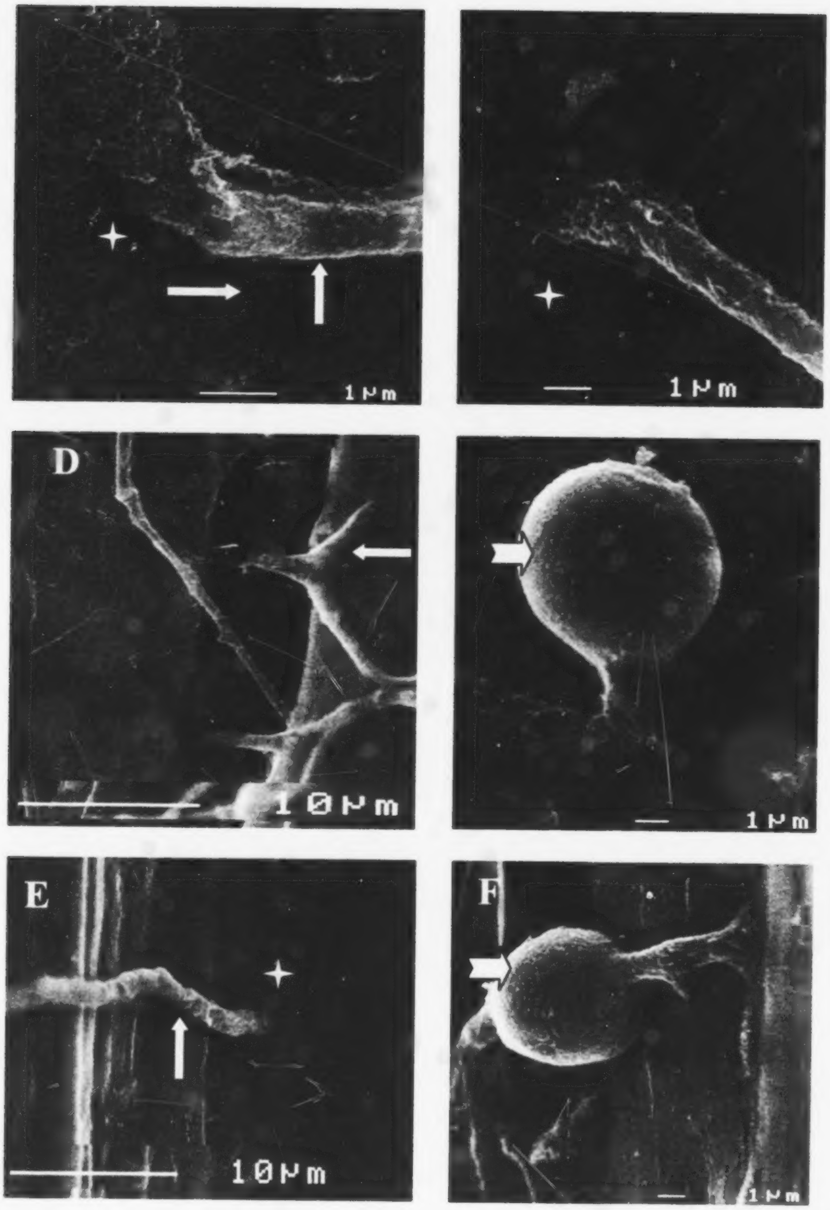
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Figure 2. Scanning electron micrograph (a-c) *Chaetomium spp.* (d-f) *Hypocrea rufa*. Note the penetration hyphae (thin arrows), infection sites (stars), and germinating conidia of *Hypocrea rufa* (thick arrows).

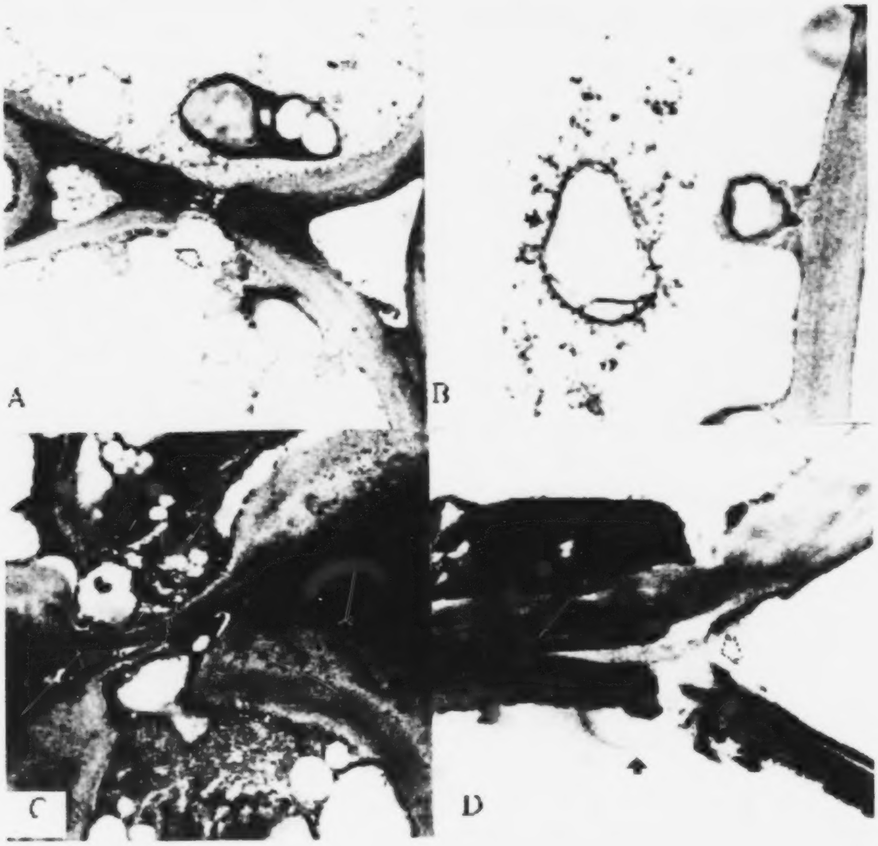

Figure 3. Transmission electron micrographs showing cellular colonisation of host (*Newtonia buchananii*) stem tissue. A, *Chaetomium spp*. note cell wall degradation (open arrow). B, dead hypha and sorrounding phenolic compounds (filled arrow). C, *Hypocrea rufa* Hypha penetration between cell walls. D, *Amphisphaeria, spp*. note Hypha penetration (filled arrow) and degradation of cell walls (open arrow).

Abiotic factors and disease occurrence

In this study, non-host seedlings (Scots pine) were incubated in Petri dishes during the whole study period. The cell death and browning of the seedling roots caused by *Chaetomium spp., H. annosum*, and *Amphisphaeria* spp. in the second 10 mm root region was slightly higher than in the first 10 mm root region at 7 days post-inoculation. This indicates that the initial invasion and penetration by the studied fungal strains was more instated in the second 10 mm root tip, suggesting that the infection was possibly above the plant root meristematic tissues. In addition, the plant incubation period could create stress conditions, which, together with the interruption by the fungi in the vascular region, could increase further stress in the seedlings by affecting water and nutrient uptake from the roots to the leaves. The penetration by the fungi through the endodermis would definitely damage the Casparian band strips, which are known to control substance apoplastic movement between cortex and stele (Asiegbu et al. 1995). The apoplastic substance pathway when the Casparian strip bands are damaged may result in toxic material entering into stele instead of the symplast pathway. Such toxic material could increase cell death and hamper nutrient and water uptake, as well as hampering the translocation of photosynthetic material in to root meristematic tissues. This suggests that the observed necrotic cell death in this study could have contributed in diminishing carbon fluxes. Water stress in plants causes a reduction in photosynthetic material, and its translocation from the leaves into the root meristmatic tips (Kramer 1983). It is well documented that quantitative resistance (QR) is very sensitive to changes in carbon fluxes, insufficient carbon assimilation or carbohydrate reserves or both (Pennypacker 2000). The tested fungal strains may have suppressed the host's quantitative resistance through hampering uptake and translocation of resources. This may explain why necrotic cell death, as a factor showing the host's ability to defend against plant pathogens (Kacprzak et al. 2001), could not resist attack by the tested fungal strains. Finally, field studies may be needed to establish the pathogenic capability of *Chaetomium* spp., *Amphisphaeria* spp. and *H. rufa* on *N.buchananii* trees.

ACKNOWLEDGEMENTS

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THE STUDY OF CHITIN-BINDING LECTIN FROM *PINUS NIGRA* SEEDS DURING THE INTERACTION OF CONIFER SEEDLINGS WITH THE NECROTROPHS *HETEROBASIDION ANNOSUM* AND *FUSARIUM AVENACEUM*

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SUMMARY

Chitin-specific lectin isolated from *Pinus nigra* seeds (PNL) was examined for its cellular localization and expression during the development of conifer seedlings after infection with *H. annosum* and *F. avenaceum*. TEM of immunocytochemical localization experiments confirmed that the seed lectin was not degraded during seed germination and development. The lectin was found to be involved in all tissues of roots, stems and cotyledons of 20-day-old pine seedlings. PNL labelling was observed on the cytoplasmic membranes and on the primary cell walls of non-infected plant cells. In the seedlings inoculated with pathogen, a interaction of PNL with the fungal cell wall was recorded at early stages of infection. At advanced stages of the infection, a much lower concentration of PNL labelling was observed, probably due to the broad damage of host cells by the pathogen or masking of PNL epitopes on the pathogen cell walls by the phenolic-like substances secreted by plants. ELISA experiments showed specific expression of PNL after exposition of *Pinus silvestris* seedlings to pathogen attack. The potential function of PNL as a recognition and defence molecule during interaction of conifer trees with necrotrophic parasites is discussed.

Keywords: lectin, pine, chitin-specific, interaction, recognition

INTRODUCTION

Lectins are a heterogeneous group of saccharide-binding proteins or glycoproteins capable of recognizing and binding carbohydrates and glycoconjugates that do not occur in plants but are abundant in the cell wall of fungi and in some structures of invertebrates (Peumans and Van Damme 1995). Because of their binding specificity, they can serve as recognition molecules within a cell, between cell or organisms. Despite the fact that their role in nature is still not clear, it is probable that lectins play fundamental biological roles in plants.

Recognition of an invading pathogen is the first step in a cascade of many chemical and structural responses. The presence of a specific recognition system in plants is supported by existence of receptor molecules localized on the cell membrane, where they are presumed to have the highest probability to detect penetrating hyphae or elicitors derived from fungal cell walls. Chitin, the basic polysaccharide component of cell walls of many fungi is known to be important in the interactions of phytopathogenic fungi with their plant hosts (Hahn 1996). Several studies were already done on identification, characterization and localization of *N*-acetylchitooligosaccharide or chitin receptors (Shibuya et al. 1993, Yamada et al. 1993). Chitin also elicits increased extracellular chitinase activity in rice cell suspension cultures and oligosaccharide elicitors from fungal cell walls serve as a signal for the induction of synthesis of a variety of defensive components in plants (Ren and West 1992).

The present study is the first on the interactions of conifer and fungi with the respect to specific expression and cellular localization of a novel *Pinus nigra* lectin (previously detected in protein bodies of European black pine seeds (Nahálková et al. 1999a) in tissues of conifer seedlings infected with necrotrophic pathogens. The potential function of PNL as a recognition and defence molecule during interaction of conifer trees with necrotrophic parasites will be also discussed.

MATERIALS AND METHODS

Biological material

Seeds of European black pine (*Pinus nigra* ARN) were collected from Arboretum, Masaryk University of Agriculture and Forestry, Brno, Czech Republic. Scots pine (*Pinus sylvestris* (L.)) seeds (319-1-1) were purchased from a plant nursery, Eksjo, Sweden. Norway spruce (*Picea abies* (L.) Karst.) seeds were gifts from Dr. Rolf Gref, Swedish University of Agricultural Sciences, Umea, Sweden. Fungal strains; *Heterobasidion annosum* (Fr.) Bref., S-type (FS6) was gifts from Dr. Kari Korhonen, Finnish Forest Research Institute, Helsinki, Finland; *Fusarium avenaceum* and *Suillus bovinus* were obtained from culture collection, Dept. of Forest Mycology and Pathology, SLU, Uppsala, Sweden. All fungal strains were maintained on Hagem agar media (Stenlid 1985).

Isolation and characterization of PNL

The protocol was based on the "glycerol method" of protein bodies isolation (Yatsu and Jacks 1968) as modified by Nahálková et al. (1999a). PNL was isolated by affinity chromatography of PB extract on 2-acetamido-2-deoxy-β-D-glucopyranoside-glycosylated poly(2-hydroxyethyl methacrylate) matrix of Spheron 300 (40-63 μm; Lachema Brno, Czech Republic) as was described previously (Nahálková et al. 1999b, 2001).

Agglutination assays

Agglutination assays were carried out in microtiter plates in a final volume of 200 μl. PNL dissolved in 10 mM phosphate-buffered saline (pH 7.2; 50 μl) was preincubated for 1h with saccharide inhibitor at the final concentration of lectin 5×10^{-6} mg/ml and total volume of mixture 150 μl. Then it was added 50 μl of 8% (v/v) suspension of washed formaldehyde fixed rat erythrocytes. Inhibition of agglutination was scored by microscopical examination after 2 h at room temperature compared to control (buffer mixed with fixed ertyhrocytes).

Serum production

Antiserum against purified *P. nigra* lectin was produced by intramuscular injection of New Zealand white rabbits as previously described (Asiegbu et al. 1993).

Seedling preparation and infection experiment

Black pine seeds were sterilized in 5% (v/v) SAVO (commercial bleach containing NaClO; Bochemie Bohumin, Czech Republic) according to Lišková et al. (1994). Scots pine or Norway spruce seeds were surface sterilised with 30% (v/v) H_2O_2 for 15 min, washed in several changes of sterile water, sown on 1% (w/v) water agar and left to germinate in the dark. Sprouting seedlings (12 to 20 days old) were used for infection studies as earlier described (Asiegbu et al. 1994, 1996a). For elicitation experiment the roots of 15 seedlings were inoculated with 1 ml of chitosan solution (0.2 mg/ml). Control was made from seedlings treated with the same volume of sterile distilled water. Samples were taken at defined time intervals and used for ELISA and immunolocalization studies.

Enzyme linked immunosorbent assay (ELISA)

Infected seedlings of *P. silvestris* were collected in certain time intervals (0-11 d.p.i.). For ELISA experiments, root extracts were prepared by homogenisation of five roots with 1.25 ml of 0.1 M carbonate buffer (pH 9.0) using a mortar and pestle. At each infection stage, three replications were used. After centrifugation for 10 min at maximum speed, the extracts (200 µl) were incubated in wells of microtiter plates overnight at 4°C. The plates were blocked (2 × 30 min) with 200 µl of 10 mM phosphate buffered saline (PBS, pH 7.2) containing 0.2% (w/v) bovine serum albumin; 2% (w/v) polyvinyl pyrrolidone and 0.05% (v/v) Tween 20 following the washing step (2 × 10 min) using PBS–0.05% (v/v) Tween 20 (PBST). Then the plates were incubated for 2 hrs at 37°C with PNL antiserum (200 µl) diluted in ratio 1:1000 with PBST containing 0.2% (w/v) BSA. After washing step with PBST–0.2% BSA–2% PVP (2 × 20 min), goat antirabbit IgG alkaline phosphate conjugate diluted 1:1000 in PBST–0.2% BSA was added and plates were incubated for 1 h at 37°C. The colour was developed according the manual of Alkaline Phosphatase EIA Substrate Kit (Bio-Rad Laboratories AB, Sundbyberg, Sweden; Cat. No 172-1063) using 100 µl of substrate solution per well. The absorbance at 405 nm was read after 20 min.

Preparation of samples for transmission electron microscopy and immunolabelling

The procedures for preparation of healthy and infected conifer seedlings for transmission electron microscopy and for immunocytochemical labelling were the same as earlier described (Nahalkova et al. 2001). The grids were examined using a Philips EM201 transmission electron microscope operated at 60kV.

Cytochemical labelling

The procedure used for cytochemical labelling was as described earlier (Asiegbu et al. 1996b). The were examined using a Philips EM201 transmission electron microscope operated at 60 kV. The specificity of labelling was assessed by incubation with the gold complexed lectin to which the corresponding inhibitory sugar (45 mM N-acetyl-D- galactosamine for HPA and 16 mM *N,N',N''*-triacetyl chitotriose for WGA) was added.

RESULTS

Saccharide-binding specificity of *Pinus nigra* lectin (PNL)

Partial saccharide-binding specificity of PNL to oligosaccharides of chitin was already demonstrated by affinity chromatography and inhibition of lectin binding to fungal cell walls (Nahálková et al. 2001). Inhibition of formaldehyde-fixed erythrocytes agglutination showed no inhibitory effect of most of the used monosaccharides – D-Glc, D-Gal, L-Fuc, D-GlcNAc, D-GalNAc at 100 mM concentration. The only positive inhibitory effect was found after addition of D-Man at final concentration of 50mM and especially pentaacetylchitopentaose which was still active at concentration 3.1×10^{-5} M.

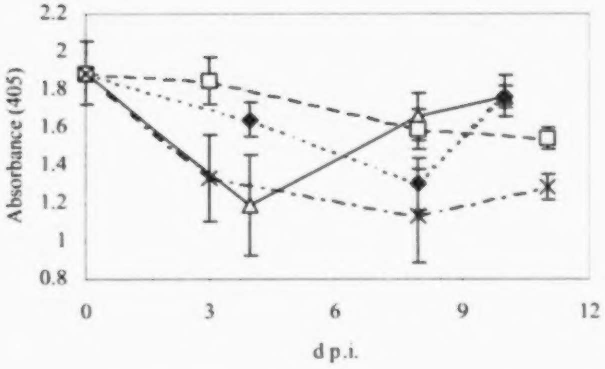

Figure 1. ELISA analysis of PNL quantity in the root extracts from 16 days old seedlings inoculated with: solution of chitosan (– –☐– –; 0.2 mg/ml); *Suillus bovinus* (- –×- –); *Heterobasidion annosum* (- -◆- -) and distilled water (control, —∇—). Error bars shows standard error of data obtained from three replications.

Expression of PNL in infected and non-infected conifer seedlings followed by ELISA method

Absorbance at 405 nm as measure of PNL concentration showed different values in extracts from roots of control plants, plants elicited with chitosan, infected with necrotroph fungus *H. annosum* and inoculated with mycorrhiza fungus *S. bovinus* (Fig. 1). At initial period after infection (0-4 d.p.i.), PNL response was higher in the extracts from roots elicited with solution of chitosan or infected with pathogenic fungus compared to control and roots treated with symbiont. At later stage of infection (8-11 d.p.i.) smaller differences between response of root extracts from chitosan elicited seedlings, control and *H. annosum* treated seedlings were recorded. On the other hand, the extract from seedlings inoculated with *S. bovinus* exhibited lower absorbance values compared to the other extracts.

Immunocytochemical localization of PNL in ungerminated and germinating *P. nigra* seeds

Immunogold labelling using monospecific PNL antiserum (Nahálková et al. 2001) and colloidal gold conjugated Protein A was used for all immunocytochemical localization studies. The results demonstrated that PNL is located both within ungerminated and germinating *P. nigra* seeds. Within ungerminated seed, PNL occurred in both the embryo and female gametophyte. Gold labelling was uniformly distributed on the cell walls of the female gametophyte cells localized directly beneath the seed coat (Fig. 2A) and on some electron dense cellular substances within the embryo (Fig. 2B). Gold particles were also observed on a large number of protein bodies within the seed tissues (data not shown). Within germinating seeds, the primary cell walls of radicle cells of the *P. nigra* seedling were intensely labelled, the labelling was found only partially on the cytoplasmic membrane (Fig. 2C). No labelling was recorded in control sections treated with preimmune sera.

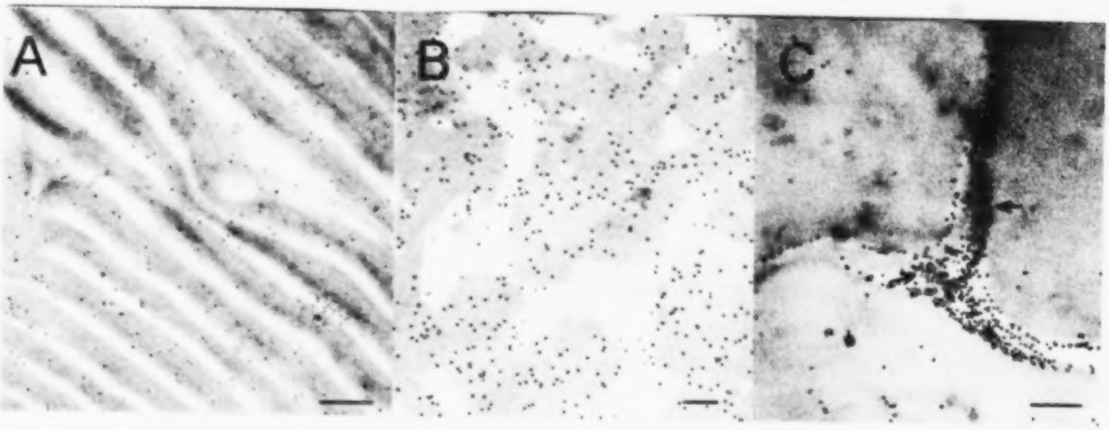

Figure 2. Cellular localization of PNL in ungerminated and germinating seeds of *P. nigra*. A, Generalized labelling of cell wall layers beneath the seed coat of *P. nigra*. B, Intense labelling of electron dense substances within the embryo of *P. nigra* seeds. C, Labelling of undifferentiated cell wall (arrows) regions of radicle in germinating seed of *P. nigra*. Bars: Figs 3A–C= 0,5 µm.

Immunocytochemical localization of PNL in roots, hypocotyls and cotyledons of 20 d old seedlings

In fully differentiated tissues of root, hypocotyls and cotyledons, the labelling occurred in all cell layers. At the subcellular level it was predominantly distributed on the plasmalemma (Figs. 3A–D) Within the cotyledons, the periphery of a number of protein bodies were labelled (Fig. 3A). No gold particles were observed on the chloroplasts (Fig. 3B). Gold labelling was recorded on the cytoplasmic membrane also in the hypocotyl region (Fig. 3C). A similar labelling pattern was observed within uninfected Scots pine root tissues (Fig. 3D).

Immunocytochemical localization of PNL in conifer tissues infected with parasitic fungi

At early stages of infection (3–5 d.p.i.) of conifer roots with the pathogenic fungi, gold particles were found not only on the host plasmalemma, but also on the fungal cell walls. Fungal cell wall labelling was intense inside of *P. nigra* root cells infected by *F. avenaceum* (Fig. 4A) and some labelling was observed also on *H. annosum* hyphae (Fig. 4B). At this stage of infection labelling very often appeared distributed around invading hyphae of *H. annosum* (Fig. 4B). To further confirm the specificity of PNL binding to the fungal chitin or chitin-related polymers, its binding pattern was compared with the binding sites of colloidal gold-conjugated wheat germ agglutinin (WGA). With WGA, gold labelling was identical to PNL localisation, i.e. predominantly on the cell walls of *F. avenaceum* (data not shown).

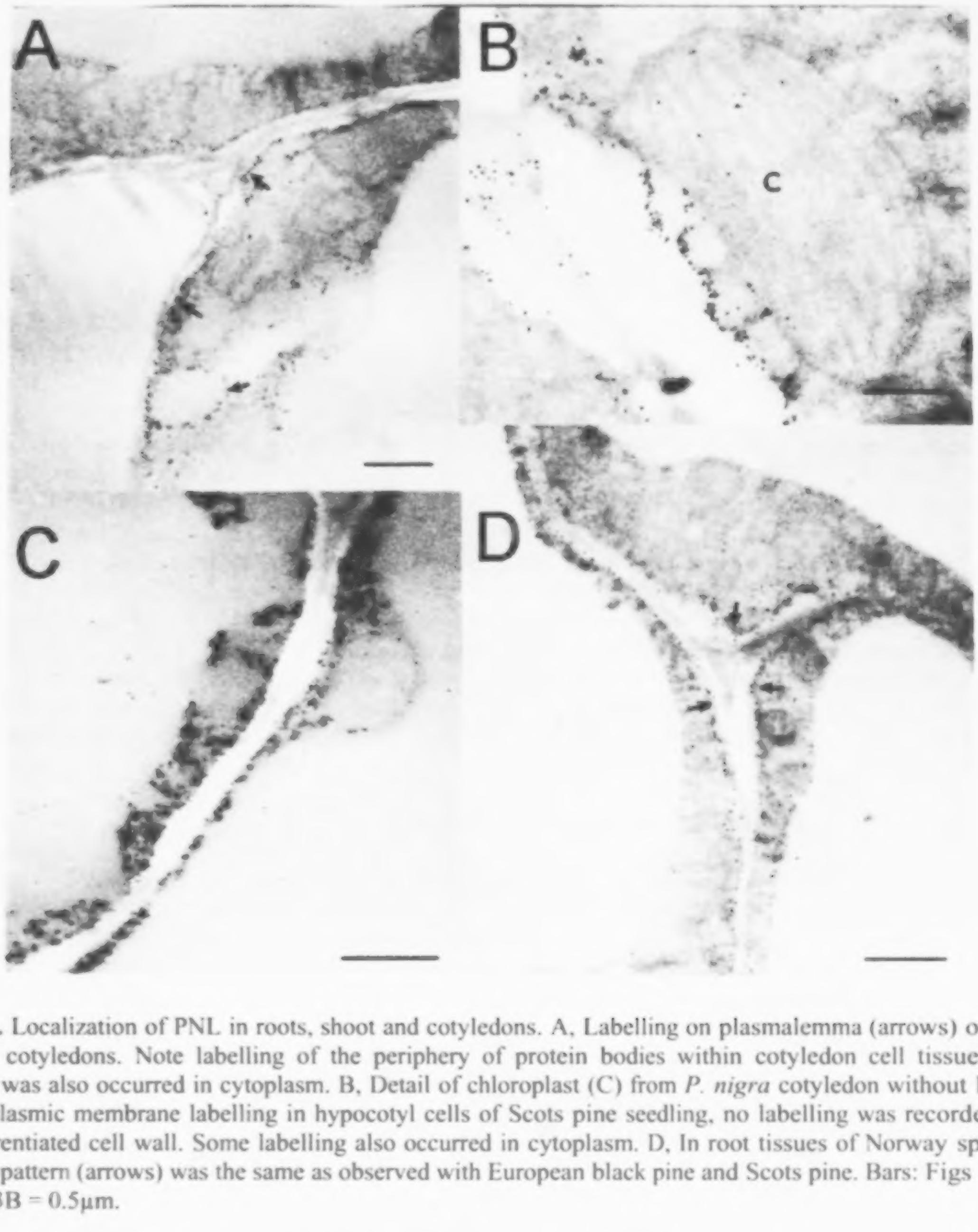

Figure 3. Localization of PNL in roots, shoot and cotyledons. A, Labelling on plasmalemma (arrows) of cells of *P. nigra* cotyledons. Note labelling of the periphery of protein bodies within cotyledon cell tissues. Minor labelling was also occurred in cytoplasm. B, Detail of chloroplast (C) from *P. nigra* cotyledon without labelling. C, Cytoplasmic membrane labelling in hypocotyl cells of Scots pine seedling, no labelling was recorded within the differentiated cell wall. Some labelling also occurred in cytoplasm. D, In root tissues of Norway spruce, the labelling pattern (arrows) was the same as observed with European black pine and Scots pine. Bars: Figs 3A, C, D = 1 µm, 3B = 0.5µm.

With progression of infection (9–11 d.p.i.), labelling of fungal cell walls was less intensive and in some cases they were almost unlabelled (Figs. 5A, 5B). Some hyphae covered with electron-dense material (probably phenolics) contained the labelling within the thick layer around the hyphae (Fig. 5C). At such advanced stage of infection, lower labelling intensity was often accompanied by destruction of the plasmalemma in the vicinity of the invading hyphae.

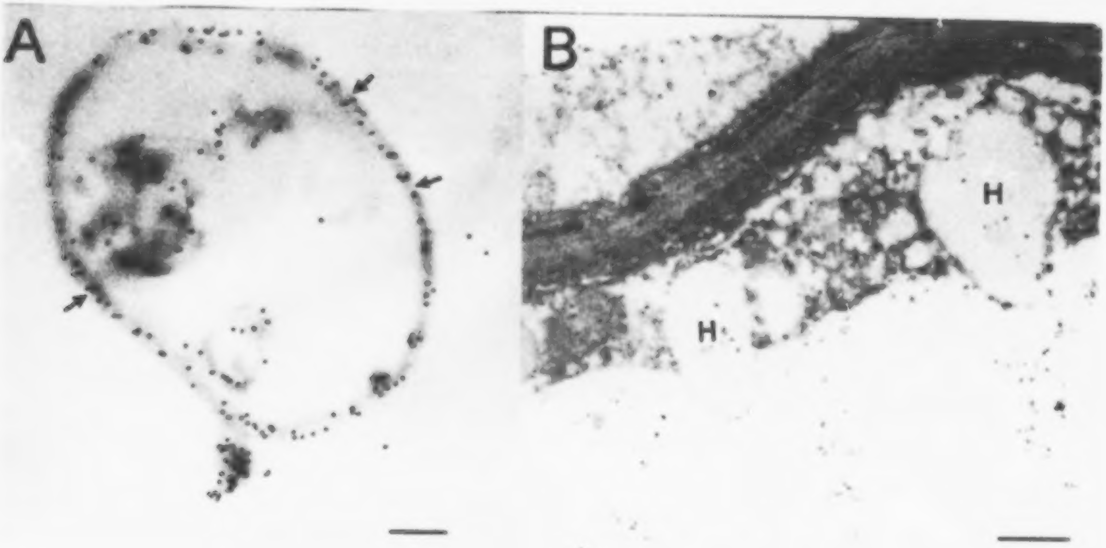

Figure 4. Localization of PNL on invading hyphae at early stages of infection. A, Intense labelling (arrow) of *F. avenaceum* hyphae within infected *P. nigra* root tissues at 5 d.p.i. Note the labelling of fungal organelles. B, Labelling (arrow) of *H. annosum* hyphae (H) within *P. nigra* root cells at 5 d.p.i. Note also the labelling distributed around the invading hyphae. Bars: Figs 4A = 0.2 µm; 4B = 0.5 µm.

DISCUSSION

The present study investigates expression and cellular localization of novel *N*-acetyl-glucosamine-specific lectin molecule isolated from *P. nigra* seeds during infection of conifer seedlings with necrotrophic parasites. Inhibition of agglutination of formaldehyde-fixed erythrocytes confirmed the saccharide-binding specificity of PNL, since pentaacetylpentaose was found as the most potent inhibitor. Inhibitory effect of D-Man seems to be not specific because inhibition was obtained at relatively high concentration (50 mM).

PNL molecule was found to be easily extractable with carbonate or phosphate buffer. Therefore, extracts from treated roots were possible to be used for evaluation of PNL expression by ELISA method. Higher PNL level in the presence of pathogenic fungi and chitosan compared to control during initial stage of infection is probably due to increased expression of PNL as a rapid response of seedlings to the presence of pathogen or saccharide elicitor molecules. The specificity of estimated PNL expression in roots infected with *H. annosum* is supported by data obtained with root extracts from seedlings inoculated with mycorrhiza fungus, where PNL quantity was very similar to control. During the second period of infection, it is possible that cell wall surface epitopes of pathogen were masked, since the difference between the responses of *H. annosum* infected root extracts and control was decreased. However, this lower difference and decreasing PNL level in the root extracts from seedlings elicited with chitosan and roots inoculated with *S. bovinus* could be a measure of PNL binding to elicitor or fungal cell walls, since ELISA method used in this study reflects only PNL quantity soluble extraction buffer.

The results of immunocytochemical localization studies indicated that PNL is generally distributed in the cells of all parts and tissues of seeds and juvenile plants. On the other hand, intracellular distribution of PNL within the tissues was found to be very specific. Some cell organelles (plasmalemma, primary cell walls, protein bodies) contained the lectin. A similar observation was made on the lectin from wheat germ (WGA), which possess a related specificity. The lectin was found in embryo and the single layer of cells that lines the interior surface of the grain coat. At the subcellular level, WGA was also found on the periphery of protein bodies and at the interface between the cell wall and cytoplasmic membrane (Mishkind et al. 1982).

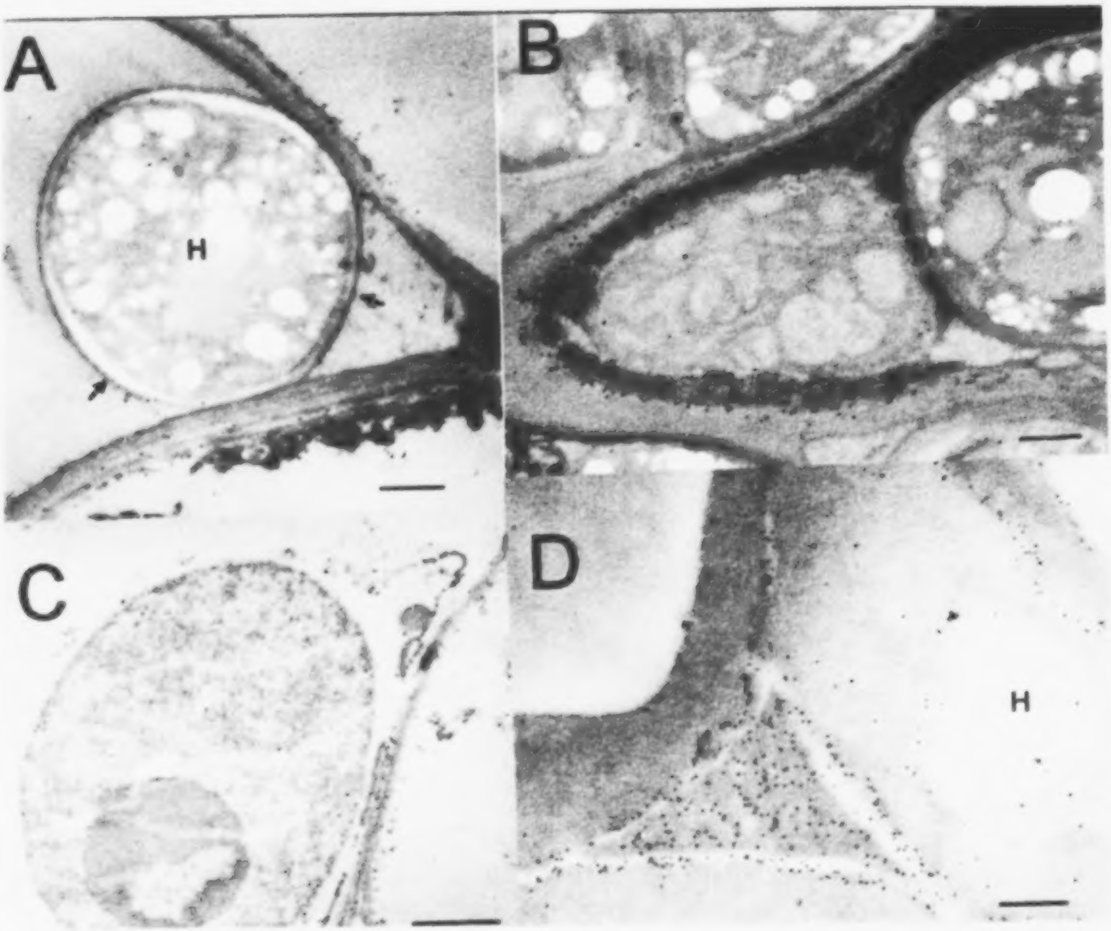

Figure 5. Localization of PNL on hyphae at late stages of infection. A, Scanty labelling (arrows) of *F. avenaceum* hyphae (H) at late stages of *P. nigra* infection (10 d.p.i.). Intensive labelling of undifferentiated cell walls and cytoplasmic membrane in the presence of pathogen. B, *F. avenaceum* hyphae covered with electron dense material within strongly infected *P. nigra* root tissues. No labelling on the cell wall of the hyphae. Note the labelling inside of the phenolic-like layers. C, Weak labelling of *H. annosum* cell wall 10 d.p.i. of *P. nigra* infection. D. Intensive labelling of undifferentiated cell walls and cytoplasmic membrane in the presence of H. annosum hyphae (H). Bars: Figs 5A, B, D = 0.5 µm; 5C = 1 µm.

Unlike many legume lectins, PNL or PNL-related molecules are not degraded after seed germination, but rather, they are distributed in all cell tissues within conifer seedlings. Furthermore, in this study, increased PNL labelling intensity was recorded in juvenile seedlings even at 27 days post germination which contrasts with results obtained in legumes where lectin levels are known to decline rapidly during seed germination (Van Driessche 1988). The consistent pattern that emerges is that PNL is located within primary cell walls of meristematic tissues and in newly emerging primordial organs (i.e. the radicle) of *P. nigra* suggesting that firstly the molecules can migrate during the process of differentiation of cell walls and then concentrate on the cytoplasmic membranes. The presence of PNL within meristematic tissues at the root tip region may indicate that it could be present extracellularly at the root surface. WGA and galactose specific lectins (from *Geodia cydonium*) have also been localized on the cell surfaces or extracellular sites (Mishkind et al. 1982, Moeramans et al. 1986).

The strong affinity of PNL to the fungal cell wall at early stages of infection strongly suggests the involvement of this molecule during initial stages of host–parasite interaction. The low hyphal labelling observed at the later stages of infection could be a result of deposition of electron-dense material on invading hyphae which prevented interaction of PNL with fungal cell walls. This idea is also supported by intensive labelling found inside of electron-dense layers covering fungal hyphae. It is also possible that electron-dense compounds secreted by plant could inhibit interaction of PNL antiserum with PNL during preparation procedure. Besides the potential recognition role of PNL or PNL-related proteins in conifer seedlings, it is equally possible that they may act directly against fungal pathogens. Recently, conclusive evidence on the existence of lectins with antifungal properties has been obtained (Broekaert et al. 1989, Van Parijs et al. 1991). Although the fungicidal properties of purified PNL are yet to be investigated, different PNL expression in elicited, infected and non-infected seedlings, specific intracellular localization of PNL on the sites of first contact of plant cells with pathogen and interaction of PNL with fungal cell walls at early stage of infection strongly suggest a functional role during host response reactions.

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EXTENT OF DECAY IN *PARASHOREA MALAANONAN* DEVELOPING FROM LOGGING INJURIES IN SABAH, MALAYSIA

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SUMMARY

Decay development associated with wounding in 40 trees of *Parashorea malaanonan* growing in Ulu Segama Forest Reserve, Sabah, was estimated seven years after logging, in compartments where reduced-impact (RIL) or conventional (CL) logging methods were used. Trees were felled and dissected to determine the volume of log occupied by decay. Scrapes were the most common types of wounds sampled (40%), followed by basal wounds (30%), broken tops (14%) or branches (6%) and butt log wounds (~5%). All wounds examined had associated decay; in contrast, stem decay occurs in about 25% of trees >30 cm DBH of this species in the Reserve. Median defect to gross volume of tree was approx. 5%, and was similar for trees in RIL and CL areas. However, defect volume per wound was greater in trees from CL relative to RIL areas; in particular, mid-bole wounds had greater defect volume in trees in CL than in RIL areas. Rate of decay was estimated at 68 cm^3 per year for each cm^2 wound area. Defect volume was positively correlated with wound size but was unrelated to tree size. Thirty fungi were isolated from decayed trees and are undergoing characterisation and identification. The results of this work will enhance our understanding of the impact of decay on *P. malaanonan* trees damaged from selective logging.

INTRODUCTION

Damage to residual trees is common during unplanned and uncontrolled selective logging (hereafter, conventional logging (CL)). In dipterocarp forests of Sabah, felling and skidding operations damage 20-70% of residual trees (Nicholson 1958; Fox 1968; Pinard and Putz 1996). The resulting wounds provide infection courts for the entry of decay-causing fungi that further reduce the potential value of the standing timber. In order to reduce the damage occurring in logging operations, reduced impact logging (RIL) has been introduced in Sabah. In general, RIL involves pre-harvest planning and vine cutting, directional felling, skidding restrictions, and post-harvest site closure (e.g., Sist 2000). Information on the incidence and extent of decay associated with wounding in dipterocarp forest is sparse. An understanding of importance of wound-associated decay to timber yields might encourage the adoption of better harvesting practices that result in less damage.

Parashorea malaanonan (local common name: Urat Mata Daun Licin) is amongst the most common commercial species occurring in southeastern Sabah. The aim of the work reported here was to quantify the extent of decay associated with wounding in *P. malaanonan* trees 7 years after logging, and to compare the extent in wounded trees in conventional and reduced-impact logging areas. This report is based on a preliminary study conducted during January-March 2000.

MATERIALS AND METHODS

Study site and tree selection: Fieldwork was conducted in Ulu Segama Forest Reserve (5° 0'N, 117° 30'E, *ca.*150-750 masl), in southeastern Sabah, Malaysia. Forty trees ($\geq$30 cm DBH) of *Parashorea malaanonan* (Blanco) Merr., 20 each from CL and RIL areas, were sampled for wound associated decay. Sampling was conducted along skid trails; sampled trees were a minimum of 100 m apart to ensure independence among samples.

Measurements of volume: Assessments of decay associated with logging wounds used methods described elsewhere (e.g., Whitney 1991; Mahmud et al. 1993). Measurements for each selected tree included DBH, total height and merchantable height (at 12 cm diameter). Stem volumes were calculated using an equation developed for *Parashorea* sp. by Forestal International Limited (1973).

Wound Measurements: Each wound was examined and data such as height above ground to the base of wound, width and length of wound, girth and stem diameter at wound, were recorded. Wound size was calculated as the product of the width and length of wound.

Discoloration and decay measurements: Boles were cut into 20-30 cm length discs, beginning at the mid-point of the wound, and continuing above and below the wound until discs were reached with no stain or decay. Each disc was dissected along a vertical plane and the boundaries of discoloration and decay marked on the exposed surface with a pen. The type of rot, brown or white, was recorded. Vertical and radial extents of the discoloured and decayed areas were recorded, and the total volume of the defect calculated.

Data Analysis: Treatment comparisons were made using t-tests (for tree dimensions) and Mann Whitney U tests (for defect / decay volumes). Spearman rank correlation analysis was used to determine associations between variables. Due to the low frequency of some types, wounds were grouped into upper (broken top + broken branch), middle (trunk scrape) and lower (butt-log wound + basal wound).

RESULTS

Characteristics of sample trees and wounds: The dimensions of trees sampled were similar in CL and RIL areas (Table 1). A total of 59 wounds attributable to logging were encountered in sampled trees, comprising 30 wounds in CL and 29 in RIL areas. All wounds were colonised by decay fungi.

Table 1. Characteristics of *Parashorea malaanonan* trees sampled from conventional and reduced-impact logging areas. Mean values presented (standard errors noted parenthetically, N = 20 per treatment).

Logging Treatment	Dbh (cm)	Merchantable height[b] (m)	Merchantable Volume[b] (m^3)
Conventional	53.3 ± 13.7	29.3 ± 7.7	4.7 ± 2.9
Reduced-impact	48.2 ± 12.7	23.7 ± 6.5	3.2 ± 2.6

[b] Up to 12 cm bole diameter

Wounds were classified into five categories (Table 2). In both conventional and reduced-impact logging areas, trunk scrapes above skidder height (> 3 m) and basal wounds were the most common type encountered; this was not surprising as trees were selected along skidding trails. Single wounds were more common (70%) than multiple wounds (30%). Trees with single wounds represented 70% and 65% of those sampled in CL and RIL areas, respectively. Wound sizes ranged from 0.005 - 1.155 m^2 (median = 0.068 m^2), and did not differ among logging treatments.

Table 2. Type of wounds on *Parashorea malaanonan* trees caused by 1993 logging operations in Ulu Segama Forest Reserve Sabah, Malaysia.

Wound type	Description
Broken top	Top of tree or trunk snapped with removal of substantial portion of crown; and tissues exposed to infection into trunk.
Broken branches	Wound at or near base of branches; branch either snapped or ruptured exposing tissue to infection into bole.
Trunk scrape	Wound or scar, ± vertical, on trunk above skidder height ($\geq$3m) into upper bole; various widths/lengths exposing sapwood.
Butt log wounds	Wounds to butt-log (buttress level up to *c.* 3m).
Basal wounds	Cankers/open wounds at stem base (ground level/buttress); large size; frequently with cavity (advanced heart rot).

Pattern of discoloration and decay development: Discoloration, typically pale brown, or yellow-brown surrounding the decay, but darker in heartwood, was present in all *P. malaanonan* trees sampled. In areas with advanced decay, heartwood tissues were fibrous and dark brown. In some trees, clear zones with brown staining were observed. White rot was present in 90% and brown rot in 20% of decay columns.

Volume of discoloration and decay: Defect volume per tree was similar among the two logging treatments (Z = 1.64, P = 0.102). Median percent stem defective for wounded trees in CL areas was 5.8% (range = 0.10 - 44%), and in RIL areas was 5.0% (range = 0.50 - 43%; Table 3). The difference between these proportions was not significant (Z = 0.663, P = 0.501). Defect volume per wound was greater in CL than in RIL (Table 3; Z = 2.138, P = 0.033). Wound position was not correlated with defect volume in either logging treatment, however, wound size was positively correlated to volume defect in both (r_{CL} =0.51, df = 30, P=0.003; r_{RIL} =0.41, df = 29, P = 0.028). Upper bole and basal wounds had similar defective volumes in the two treatments but mid-bole wounds had greater defective volume in CL relative to RIL areas (Figure 1). By pooling the volume of defects in all samples, the rate of decay was estimated at 68 cm^3 per year for each cm^2 wound area.

Table 3. Volume (m^3) of discoloration and decay (i.e., total defect) associated with wounds in *Parashorea malaanonan*. Thirty and 29 wounds were sampled in 20 trees in conventional and reduced-impact logging areas.

Logging Treatment	Volume of total defect per wound	Volume of total defect per tree
Conventional	0.281; 0.002-3.06	0.310; 0.002-3.493
Reduced-impact	0.092; 0.031-1.12	0.140; 0.010-1.116

Note: Values are medians, followed by extremes.

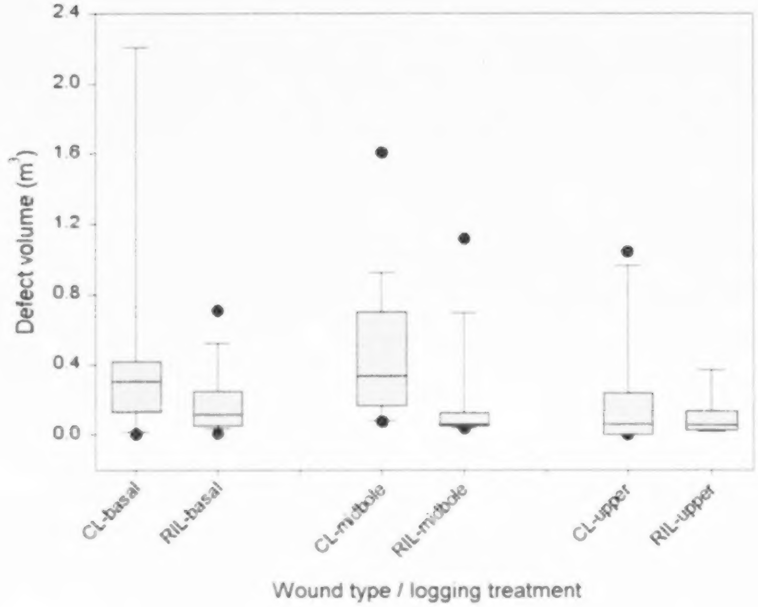

Figure 1. Volume of defect in three height categories in *Parashorea malaanonan* trees sampled in conventional (CL) and reduced-impact (RIL) logging areas. Upper wounds include broken tops and broken branches; mid-bole wounds include trunk scrapes; basal wounds include butt-log wounds and basal wounds.

DISCUSSION

Decay and discoloration was found in all wounds examined on *P. malaanonan* trees in the logged forest. The general incidence of stem decay in undamaged trees of this species is about 25% (Sudin, unpublished data), suggesting that logging wounds substantially increase the incidence of decay in this species. Seven years post-wounding, median stem volume losses to decay were about 5-6%; over a 30 year cutting cycle, however, stand-level losses due to wound-induced decay could be substantial. Pooled data indicated that decay increased at a rate of 68 cm^3 per cm^2 of wound area per year. Decay may expand slowly soon after wounding, but increase rapidly, as the fungus becomes more established (Hennon and Demars 1997). Concern over longer-term yield losses relates to both conventional and reduced-impact logging areas: although incidence of wounding is much less in RIL than CL sites (Pinard and Putz 1996), the likelihood of wound-related decay seems similarly high.

Our results suggest that the rate of decay development in mid-bole wounds (i.e., trunk scrapes) may be greater in CL relative to RIL areas. Contrasting post-logging environmental conditions may be responsible for differences in tree growth rates, and trees' responses to wounding and infection. Conventional logging sites are typically more open and drier than RIL areas, at least for the first 10 yr post-logging (Pinard, personal observation). The volume of decay may also vary with the species of fungus responsible, and be influenced by the ecological strategies of the fungi. Some species are adapted for early colonization (ruderal strategists), whereas others are effective competitors against others species present in the niche (stress-tolerant strategists) or are dominant invaders or defenders of the domain (combative strategists) (Rayner and Boddy 1988).

Multiple wounds were slightly more frequent on trees sampled in RIL relative to CL areas. While this study was not designed to compare wounding types or incidence in the two treatments, the similarity we observed between treatments in our sample concurs with the finding of Yap et al. (1993) who assessed logging damage in Ulu Segama and found that severity of damage was similar on damaged residual trees in the two treatment areas. The classification system used here (Table 2) for wound types is consistent with those used by others (e.g., Nicholson 1958; Whitney 1991). The predominance of trunk scrapes and basal wounds in our sample is more likely to be an artefact of the method of tree selection (along skidding trails), than a representation of relative incidence across logged forest. Indeed, Nicholson (1958) and Pinard and Putz (1996) recorded broken branches and snapped stems as the most common type of damage in eastern Sabah.

Typical white rot decay was observed in most of the decayed wood examined. Similar observations were also reported in *Acacia mangium* in Sabah (Sudin et al. 1993). Discolored wood in the reaction zones surrounding decay generally retains structural strength, and for some species may still suitable for pulp (Hennon and DeMars 1997). The position of the discolored wood, contiguous with the decay, however, increases the volume of defect. Thirty fungi were isolated from the decayed trees, including a *Phellinus* sp., the identity of which is currently under investigation.

This study has provided evidence that decay developing from wounds may cause substantial defects and reduce the quantities of useable timber in dipterocarp forests, at least for the common species, *P. malaanonan*. Improving harvesting such that incidence of wounding is reduced should result in greater recovery of timber in subsequent harvests.

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PHENOLOGICAL CHANGE AND DISTRIBUTION OF BASIDIOCARPS OF *PHAEOLUS SCHWEINITZII* IN A SEVERELY INFECTED LARCH STAND

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SUMMARY

The phenology of the basidiocarp development of *Phaeolus schweinitzii*, which causes brown cubical butt rot in commercial timber species of living conifers, was investigated in a larch stand. During the three years of observation, the development of the basidiocarps started in the middle of June. The number of newly formed basidiocarps increased in July and August. The development was even observed at the end of September. During the basidiocarp season, active basidiocarps that had the ability to release basidiospores were always present. Analysis of the distribution pattern showed that the basidiocarps were distributed in small clumps. Comparisons with an unthinned stand suggested that thinning encouraged the development of basidiocarps.

Keywords: *Phaeolus schweinitzii*, phenology, spatial distribution, butt rot, thinning

INTRODUCTION

Phaeolus schweinitzii (Fr.) Pat. is considered to be the major cause of brown cubical butt rot in commercial timber species of living conifers in northern hemispheres, including cool-temperate to boreal forests in northern Japan. The basidiocarps of the fungus are annual, usually developing from summer to autumn. They appear on the ground from roots or on the base of living trees, stumps, or logs (Gilbertson and Ryberden 1987). The basidiospores of *P. schweinitzii* are effective in initiating a persistent saprophytic infestation of soil (Barrett 1985). However, little is known about the ecology of the fungus, especially the phenology of basidiocarp development. This paper describes the seasonal change of the basidiocarp development of *P. schweinitztii* and its distribution in a larch stand where quite a high incidence of butt rot caused by the fungus was observed after thinning.

MATERIALS AND METHODS

The study was conducted in a Japanese larch plantation in an experimental forest of the Hokkaido Research Center at the Forestry and Forest Products Research Institute, Sapporo City, Hokkaido, northern Japan. The stand was 23 years old when the study was started, and the area of the stand was about 0.35 ha. Prior to the research, thinning had been carried out in the winter of 1997, and about 19% of the total number of trees had been cut. When thinning was conducted, butt rot was found in 59% of the total thinned stumps, and *P. schweinitzii* was isolated from 45% of butt-rotted stumps.

The number and position of newly developed basidiocarps in the stand were noted at intervals of 7 to 20 days from June to October in 1998, 1999, and 2000. The location of each basidiocarp was recorded on the map of the stand. The vitality of developed basidiocarps was assessed by the color of the pore surface. To evaluate the effect of thinning, the number of basidiocarps developed in an unthinned stand just next to a thinned stand was simultaneously counted. The distribution pattern of individual basidiocarps was analyzed by using the I_δ method (Morishita 1959).

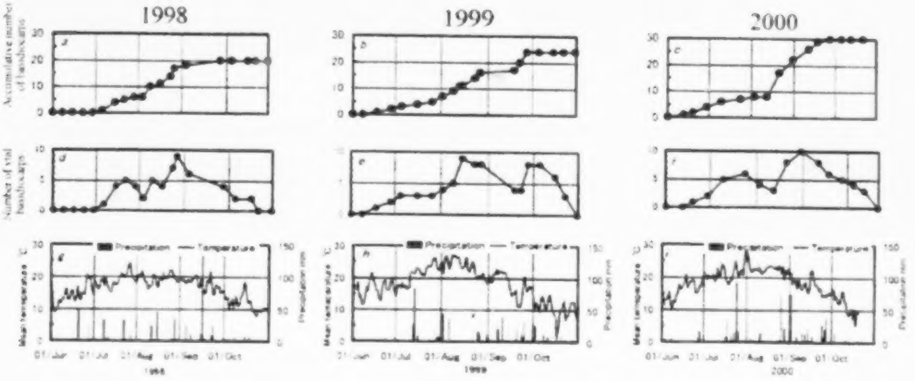

Figure 1. Seasonal changes of accumulative number of newly formed basidiocarps (a, b, c), number of vital basidiocarps (d, e, f), and mean temperature and precipitation (g, h, i) throughout the three-year observation.

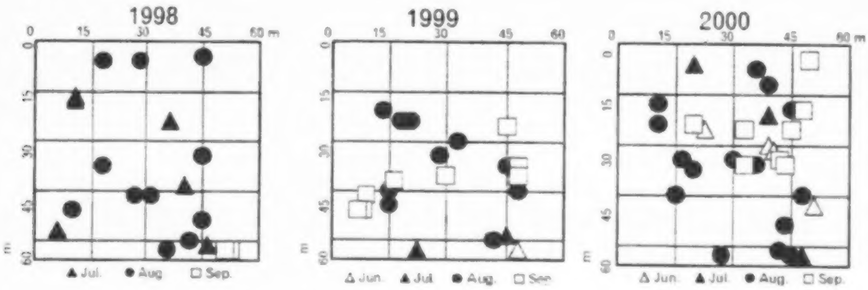

Figure 2. Spatial distribution of basidiocarps of *P.schweinitzii* and their seasonal changes.

Table 1. Number of basidiocarps on the stumps or on the base of a living larch and effect of thinning on the development of basidiocarps.

Position of developed basidiocarps	Thinned stand			Unthinned stand		
	1998	1999	2000	1998	1999	2000
On the surface of thinned stump or base of the stump	14	13	16	-	-	-
On the base of a living larch	6	10	13	2	1	2
Old stumps (previously cut)		1	1	-	-	-
Total	20	24	30	2	1	2

RESULTS

During the three years of observation, the development of the basidiocarps of *P. schweinitzii* started in the middle of June in 1999 and 2000 (Fig.1). In 1998, the first development of basidiocarps was observed in early July. The number of newly formed basidiocarps considerably increased in July and August, as the temperature rose. Basidiocarp development was even observed at the end of September in 1999 and 2000. Individual basidiocarps were ordinarily active in two to three weeks. Vital basidiocarps that had the ability to release basidiospores were continuously present in the stand from June or July to September.

Analysis of the distribution pattern showed that the basidiocarps were aggregated in small clumps in the stand, especially in 1998 (Fig.2). However, the aggregated pattern was less typical in 1999 and 2000, as the year passed after thinning. Thus, the distributional pattern was different in each of the three years. Seasonal changes in the pattern of developed basidiocarps also varied from year to year within the observation period, showing no apparent tendency (Fig.2).

A remarkable number of basidiocarps occurred on the thinned stand in comparison with the unthinned one (Table 1). Most basidiocarps were formed on the thinned stumps or near the stumps which were cut at thinning, while some basidiocarps developed at the base of a living larch.

CONCLUSION

Our three-year observation showed that the period of basidiocarp development and spore dispersion in *P. schweinitzii* was quite long in the study site (Fig.1). The basidiospore of *P. schweinitzii* seems to have an important role in the spreading and colonization of *P. schweinitzii* in forests (Barrett 1985); however, the mode of vegetative spread and infection of the fungus is still unknown. The long period of spore dispersion and aggregated basidiocarp development may be advantageous for the spread and infection of the fungus.

Comparisons with the unthinned stand suggested that thinning encouraged the development of basidiocarps (Table 1). In general, nutrition, light, gaseous regime, humidity, temperature, physical or chemical stimulation, and other factors are known to induce the development of the reproductive stage (Rayner and Boddy 1988). Thinning may change the conditions of light, temperature, and humidity in the stand, while tree cutting causes nutrition changes and physical disturbance. These factors may enhance the formation of the basidiocarp of *P. schweinitzii*. Thinning in such a heavily infected stand should hence be done more cautiously because the development of basidiocarps and spore dispersal may be enhanced by thinning.

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HETEROBASIDION ROOT ROT – A THREAT TO THE FORESTS IN ESTONIA

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SUMMARY

In this paper, the current knowledge concerning the distribution of the *Heterobasidion* root rot in the forests of Estonia is studied, with a special reference to men's silvicultural activities in forests. Estonia is one of the first countries to accept the new nomenclature of the pathogen, treating former S- and P-types of *H. annosum* s.l. as *H. parviporum* and *H. annosum* s.str. The distribution of both species of *Heterobasidion* in different forest stands of Estonia and the rate of post-thinning infection of Norway spruce (*Picea abies*) stands in South Estonia are discussed. The most endangered forest site types for Norway spruce and Scots pine (*Pinus sylvestris*) are specified. Some speculations are posed concerning the different role of the disease in current and pre-silvicultural era.

Keywords: root rot, *Heterobasidion* species, distribution

INTRODUCTION

Among several other tree diseases, *Heterobasidion* root rot has accompanied its hosts for several tens of thousands of years. The retrospection of the history of *Heterobasidion* root rot has already reached the period of the natural (re)forestation of the northern part of Europe after the last glacial period (Johansson and Unestam 1988). During most of this long period, *Heterobasidion* root rot has played an unknown role in the life of natural forests. Its well-known large-scale devastating action to forest trees has been limited by the two last centuries, i.e. by the time of the fast development of men's activities in forests, including the role of silviculture as a science and action. "*H. annosum* is a fungus that follows man's footsteps into the forest" (Korhonen et al. 1998).

LIST OF HOSTS AND TAXONOMY OF THE PATHOGEN

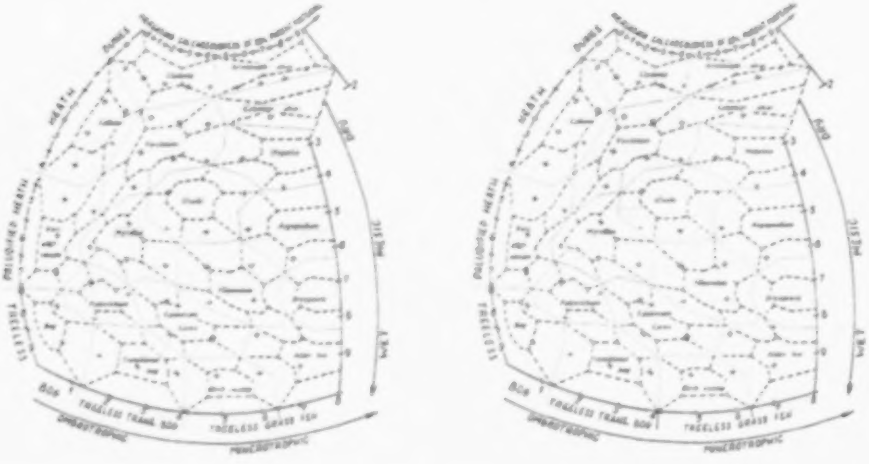
W.A. Sinclair (1964) counted nearly 150 species of plants (trees, bushes and ground vegetation) known to be attacked by *Heterobasidion* root rot. In the course of the forest pathological research in Estonia, we have diagnosed the disease on 12 host species (conifers, broadleaved trees and bushes). No attempts were made to increase the number of hosts by any special investigation. Therefore, there have to be much more hosts to *Heterobasidion* in our country.

The genus *Heterobasidion* Bref. (*Coriolaceae, Poriales*) was known to include six distinct taxonomic species. Investigations of different researchers have revealed that the old aggregate species *Heterobasidion annosum* (syn. *Trametes radiciperda, Fomes annosus*) actually consists of three to five intersterility groups, three of them occurring in Europe and two in North America. In a book summarizing knowledge about *Heterobasidion* root rot (Woodward et al. 1998), the name *Heterobasidion annosum* (Fr.) Bref. was proposed for the so-called P (pine) group, *H. parviporum* Niemelä & Korhonen for the S (spruce) group, and *H. abietinum* Niemelä & Korhonen for the F (fir) group of isolates. Estonia was one of the first countries where this new nomenclature was accepted.

As a result of special investigations (Hanso and Hanso 1999), we have found here two of the *Heterobasidion* species - *H. annosum* and *H. parviporum*. The first occurs mainly on *Pinus sylvestris, Juniperus* *communis* and native hardwood species; the second on *Picea abies* and on some introduced conifers. By today, approximately 500 pure cultures of *Heterobasidion* have been isolated from Estonia. Estonian isolates have been used, together with some other strains, in the molecular differentiation of *H. abietinum* as a new species in Italy (Capretti et al. 1990) and later on in the diagnoses of the occurrence of this new species in Poland (Łakomy 1996).

DISTRIBUTION

Frequency of occurrence of the disease depends on natural as well as anthropogenic factors. The forests most susceptible to *Heterobasidion* root rot in Estonia grow on site types which are indicated by us (Hanso and Hanso 1999) on the basic ordination scheme compiled by E. Lõhmus: severely are attacked Norway spruce (*Picea abies*) stands growing on *Oxalis* and some neighbouring (cf. the scheme, Fig. 1) forest site types, and Scots pine (*Pinus sylvestris*) stands growing on *Vaccinium* and neighbouring site types, extending preferably to the warmer and dryer than to the colder and wetter neighbouring types (Karu 1953; Muiste 1959, 1965; Hanso and Hanso 1999).



A

B

Figure 1. Forest site types with high risk of *Heterobasidion* root rot for *Picea abies* (A) and *Pinus sylvestris* (B).

Forests located on former non-forested lands (first generation stands) are always more susceptible to *Heterobasidion* root rot than forests located on permanent forest lands. Distribution of the two *Heterobasidion* species on different hosts in different forests of Estonia can be seen in Table 1.

Table 1. Occurrence of *Heterobasidion parviporum* (S) and *H. annosum* (P) in different forest stands in Estonia.

Forest	Number of isolates	Host tree							
		Spruce		Pine		Juniper		Deciduous	
		S	P	S	P	S	P	S	P
I Stand on permanent forest lands									
Spruce forests	80	73	1	0	4	1	0	0	1
Pine forests	69	8	0	0	36	0	16	0	9
Mixed stands	158	109	1	0	17	1	12	2	16
Altogether	307	190	2	0	57	2	28	2	26
II Stands on non-forest lands (former arable lands, afforested oil-shale open-mine areas)									
Spruce forests	78	78	0	0	0	0	0	0	0
Pine forests	45	0	0	0	39	2	0	0	4
Mixed stands	22	0	1	1	5	0	0	0	0
Total	145	78	1	1	44	2	0	0	4

In comparison with nearby countries where similar investigations have been carried out (Finland, Lithuania, Denmark), there is less *H. annosum* (P-type) on spruce in Estonia (Table 2).

Table 2. Frequency of *Heterobasidion parviporum* (S) and *H. annosum* (P) on main hosts in Finland (Korhonen and Piri 1994) and in Estonia (Hanso and Hanso 1999).

	In Finland			In Estonia		
Host tree	**Number of isolates**	**S %**	**P %**	**Number of isolates**	**S %**	**P %**
Picea abies	1 176	89	11	286	98	2
Pinus sylvestris	499	19	81	102	1	99
Juniperus communis	67	7	93	32	6	94
Betula spp.	31	6	94	11	0	100

Forest thinning is the most investigated anthropogenic factor in connection with the distribution of *Heterobasidion* root rot. Nowadays, professional workers cut trees with no regard for the season, which is different from the earlier practice when cuttings were done by farmers who only had time to do so in winter. Stump surfaces during the summer cutting serve as gates for the spore infection by *Heterobasidion*, which ends with the formation of gaps of dead trees by the infection of neighbouring growing trees through root contacts. Some data concerning post-thinning infection of Norway spruce stands on experimental areas in South Estonia are indicated in Table 3. After cutting in the course of a thinning operation of an infected but still alive tree, the vegetative growth of the pathogen in its (stump's) roots even accelerates.

Table 3. Rate of infection of Norway spruce stands by *Heterobasidion* root rot on the old thinning experimental areas in South Estonia.

Sampling area	**First thinning year**	**Stand age at the time of first thinning**	**Number of thinning successions**	**Abundance of trees (%) with *Heterobasidion* root rot in 1998**		
				Intensive thinning	**Moderate thinning**	**Unthinned**
Rokka	1953	18+2	2x	81	62	0
Kihu	1953	16+2	2x	56	24	/24/*
Punasoo	1951	14+2	3x	30	17	0
Kokemae	1962	15+4	1x	20	13	0
Palupera	1955	10+4	3x	47	20	0
		Average:		46.8	27.2	0/4.8/

* a moderate thinning has been carried out later on.

Compared with the transfer of the pathogen to the same host species during the distribution process through root contacts, host change hinders the rate of infection. It may be one of the reasons why admixtures of tree species (i.e. the mixed forests) are, as a rule, more healthy.

HETEROBASIDION ROOT ROT IN THE SUCCEEDING FOREST GENERATIONS

After it has infected a forest stand and caused permanently growing gaps of dead trees, *Heterobasidion* survives the final cutting in stumps and roots and may continue destruction in the next forest generation. Large-scale forest fires, one of the possible natural factors eliminating or reducing the source of *Heterobasidion* infection from soils of the warmer and dryer forest site types during the era of natural forests, are rare or absent in managed forests nowadays. Infection, as a rule, accumulates and causes growing trouble.

The diversity of natural forests is the feature which allows us to speculate that before silviculture – as a science and action – the threat from *Heterobasidion* root rot was much smaller. It is also silviculture, in collaboration with the other disciplines involved (forest pathology, forest industry, environmental sciences, etc.), that has to solve this problem.

ACKNOWLEDGEMENTS

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COMPARATIVE STUDY BETWEEN NORWAY SPRUCE AND SILVER FIR TREE HEALTH STATUS INFECTED BY *HETEROBASIDION ANNOSUM* USING ELECTRICAL RESISTANCE AND CHEMICAL COLORATION

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SUMMARY

This paper summarizes the main results obtained in NP Durmitor (UNESCO, M&B), Montenegro, on *Heterobasidion annosum*, a causal agent of a root rot of Norway spruce and Silver fir. Based on the cambial electrical resistance (CER) and chemical coloration analyses, this investigation confirmed a correlation between *H. annosum* attack and detecting changes in trees health and productivity.

Keywords: Forest decline, root rot, *Abies alba*, *Picea abies*, NP Durmitor

INTRODUCTION

Silver fir (*Abies alba*) and Norway spruce (*Picea abies*) trees in mature forest of NP Durmitor (UNESCO, M&B), Montenegro, south-eastern Europe, have maintained high mortality in recent decades (Vujanovic 1995). The precise aetiology of declining tree health (Figs. 1 and 2) is not know, but air pollution and climatic changes (Vujanovic 1994) in association with *Heterobasidion annosum* (Fr.) Bref. (Fig. 3) infections (Karadzic and Vujanovic 1994) are considered as the most important problem. The objective of this study is : to determine the relationship of cambial electrical resistance (CER) with detecting changes in trees health and productivity in correlation with *H. annosum* attack.

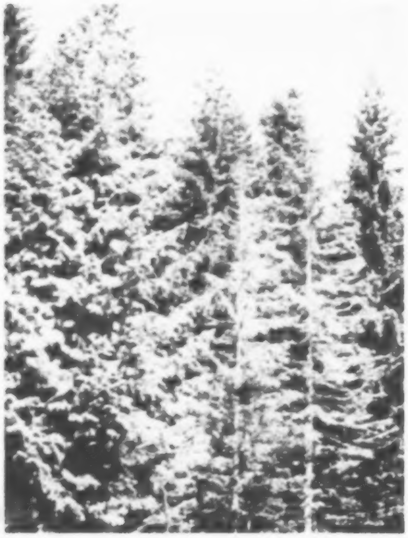


Fig. 1. Crown chlorosis.

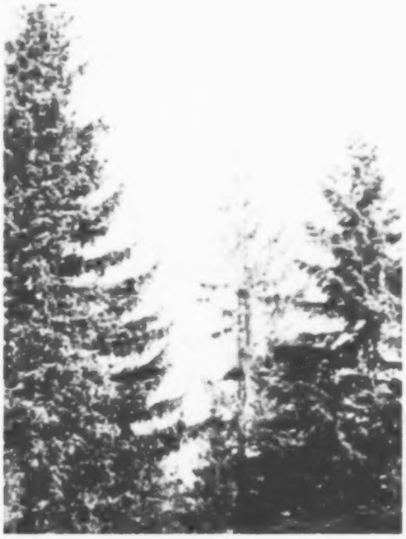


Fig. 2. Trees die-back.

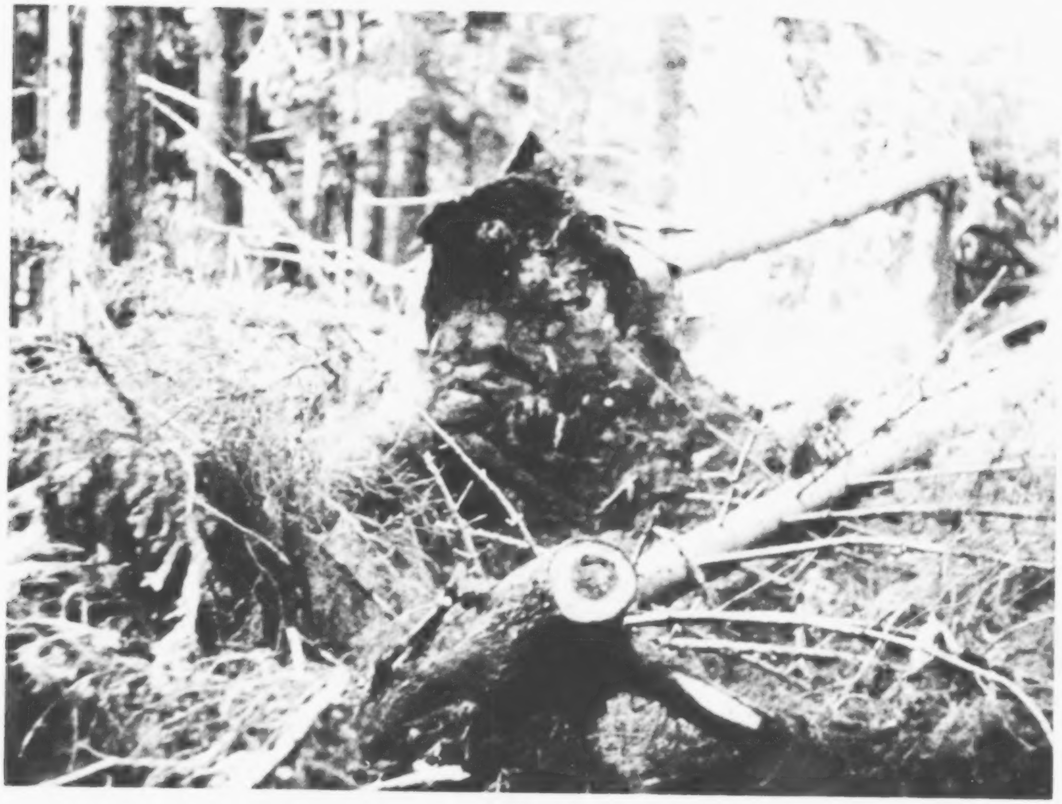


Fig. 3. Forest damage caused by simultaneous impact of *Heterobasidion annosum* (arrow) and wind.

MATERIALS AND METHODS

Research was conducted in NP Durmitor, Montenegro, Yugoslavia, on two localities: Razvrsje (Ass. *Picetum)* and Mlinski Potok (Ass. *Abietum*), 1400m asl. Using a cambial electrical resistance (CER) and crown density index (CDI) (Vujanovic 1995), trees were assigned in various vitality and vigour categories (0+1 healthy trees and 2+3 declining trees). For these categories, three spruce and three silver fir trees were chosen in each of four stem heights (h = 6 m, 8 m, and 12 m and 24 m) to inspect the presence of the *H. annosum*. Then, the wood samples were taken with Pressler borer at h = 0.3 m towards the centre of stem. Diseased trees were cut in order to measure decay intensity [a) decay and b) discoloration)] and distribution caused by *H. annosum* (Fig. 5). Control samples were taken to isolate this fungus on agar media. CER was measured in kΩ by CONDITIOMETAR (Fig. 4) and discoloration was estimated staining wood by cotton-bleu aqueous suspension (0.1%) (Fig. 5). CER data were collected during August, and they were corrected to 15°C using the following equation (Bauce 1989): adjusted CER = CER + 12.99 – T / 0.0927T-0.236.

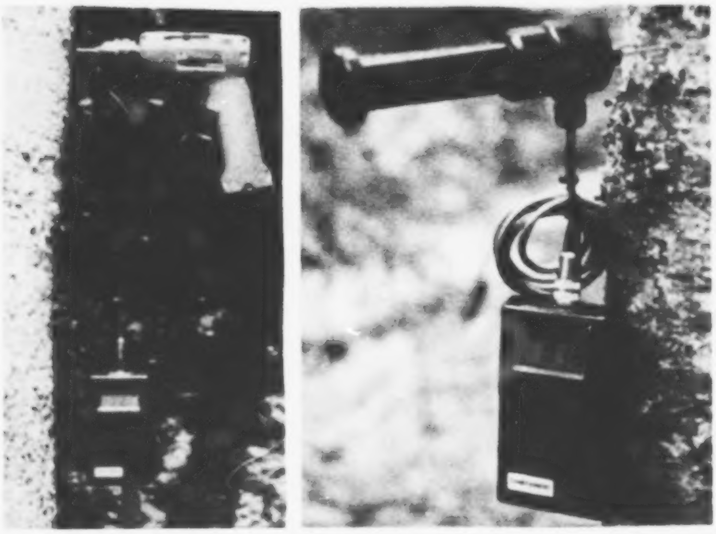


Fig. 4. Conditiometer-AS1 (Bollman Electronic-System, Rielasingen, Germany) equipped with two electrodes pins, one probe, and MAKITA-1612D borer.

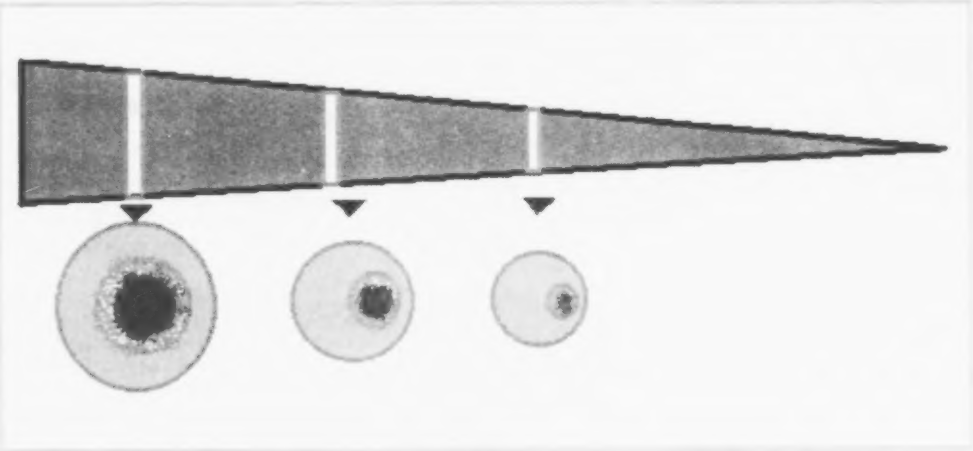
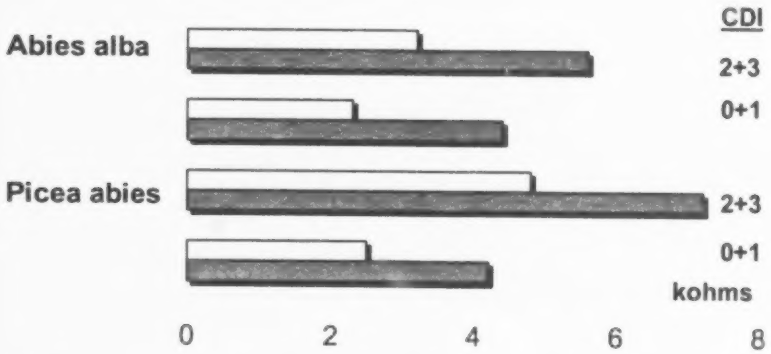


Fig. 5. Cut stem was sectioned at three different heights: - *Tree base* (0.3 m); - *Breast height* (1.3 m); - *Mid-stem* and then each section was sprayed by cotton-blue.

RESULTS

CER was significantly ($p<0.05$) higher in i) young trees than in mature trees, ii) decaying trees than in healthy trees, and iii) Norway spruce than in silver fir (Fig. 6.).



CER (kohms)	P. abies (0+1)	P. abies (2+3)	A. alba (0+1)	A. alba (2+3)
□ d1,3 > 30 cm	2,5a	4,8 b	2,3 a	3,2b
▨ d1,3 < 30 cm	4,2a	7,2 b	4,4 a	5,6b

Fig. 6. Relationship of CER at breast height (1.3 m) with changes of CDI observed in young and mature trees of *P. abies* and *A. alba*.

Fungal colonization was relatively greater in young (H < 12 m) than in mature (H > 12 m) declining trees (CDI = 2+3) and in the proximal (towards the bole) than in the distal direction of stem (Fig. 7).

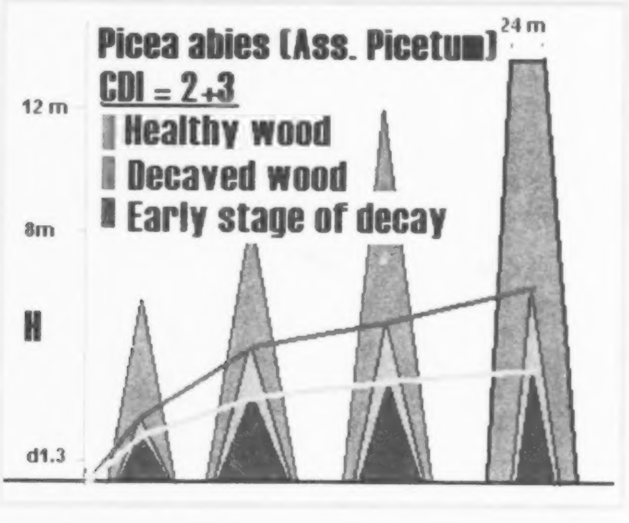

Picea abies (Ass. Picetum)
24 m
CDI = 2+3
Healthy wood
Decayed wood
Early stage of decay
12 m
8m
H
d1.3

Fig. 7. *H. annosum* distribution is visible by purple-brown staining in decayed wood with early stage of decay becomes visible when staining in cotton-blue.

Decay in *P. abies* trees was significantly higher than in *A. alba* showing less trees health and productivity in forest association *Picetum* than *Abietum* (Table 1).

Table 1. Comparison between mean values of basal area (BA) and the decayed-wood area (DWA) in trees of Norway spruce and silver fir (CDI:2+3) infected by *H. annosum* (*HA*).

Forest association	**Tree base (0.3m)**		***HA* decay**	**Breast height (1.3 m)**		***HA* decay**
	BA	**DWA**	**%**	**BA**	**DWA**	**%**
	(cm²)			**(cm²)**		
Ass. *Picetum* (loc. Razvrsje)						
Picea abies	82*a*	52*a*	63	56*a*	26*a*	46
Abies alba	80*a*	31*b*	39	53*a*	6*b*	12
Ass. *Abietum* (loc. M. Potok)						
Picea abies	84*a*	51a	61	68*a*	27*a*	39
Abies alba	78*a*	20*b*	25	47*a*	14*b*	3

Note: Within each line, identical letters indicate that mean values are note significantly different ($p<0.05$) by Duncan's test of multiple mean comparison.

DISCUSSION AND CONCLUSION

In this study, changes in tree health and productivity have been correlated with *H. annosum* attack as previously has been done in the other regions of Europe (Lindberg 1991) and North America (Filip et al. 2000). Both CER and CDI were inversely correlated up to advanced stages of the decay in both two hosts (*Picea abies* and *Abies alba*). Variation in CER and decay intensity showed a relative tolerance of silver fir towards fungal invasion compared with Norway spruce sensitive reaction. CER has also been found to correlate with the tree diameter at breast height and its visual crown appearance (Lindberg and Johansson 1989, Stefancik 1997, Tomiczek 1995). The results show low CER values, which could be partially explain with high moisture and temperature that occurred within the tree's assessment process. Early detection of CER and CDI could be helpful for forest managers in order to reduce the problem of *H. annossum* on declining coniferous forest stands. Future investigations is needed to distinguish : various *H. annosum* intersterility groups (ISGs: S-, P-, and F-types) in large region of the NP Durmitor (Worrall et al. 1983), its associated host specificity (Sullivan et al. 2001) and CER values.

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PRELIMINARY EVALUATION OF SCOTS PINE PLANTATIONS "RESISTANT" TO *HETEROBASIDION ANNOSUM* BREF. (FR.)

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SUMMARY

The present study was carried out in experimental plantations of *Pinus sylvestris* L. established on the former agricultural land in Central Lithuania, in 1975. In those plantations, thinnings were meant to be delayed for the longest time possible, thus avoiding damage by *Heterobasidion annosum*. Four different planting schemes have been used. Earlier work has shown that some nitrogen-fixing trees (*Robinia pseudoaccacia* L.,*Amorpha fruticosa* L.) have antagonistic properties to *H. annosum in vitro* (Vasiliauskas et al. 1976). The main aims of the study were to evaluate growth characteristics of these stands, to examine the effect of deciduous tree species mixed with Scots pine and to search for possible infections by *H. annosum*. The number of tree and stand parameters were assessed by direct measurement or visual evaluation. Sampling of stems and isolation of fungi were carried out as described by Vasiliauskas et al. (1996). Wood samples were taken at the root collar of 152 *P. sylvestris* trees in two planting schemes. Analysis of field data showed a negative correlation between the current density of trees and the general productivity of stands investigated. Pure stands of *P. sylvestris* (set as control) had the highest density; however, the productivity factors were the lowest. On the contrary, mixed *P. sylvestris* – *A. fruticosa* stands provided reverse results, showing the highest quality from those points of view. No isolates of *H. annosum* or other *Basidiomycetes* have been grown out from the wood samples, whether it be from sound looking or from declining pines. In conclusion, stands in the schemes I-III have an acceptable density at the age of 25 years and still do not require thinning (1). No part of the stands showed any signs of root rot attack, although some trees are declining due to suppression. *P. sylvestris* trees were mostly inhabited by endophytic fungal species (2).

Keywords: *Heterobasidion annosum* root rot, *Pinus sylvestris*, tree and stand characteristics, thinnings, density of stands

INTRODUCTION

Heterobasidion annosum (Fr.) Bref. is one of the most important pathogens in boreal forests, causing root and butt rot on many tree species, especially conifers. The fungus enters Scots pine (*Pinus sylvestris* L.) stands mainly through the stumps made during thinnings (Rishbeth 1951; Chase and Ullrich 1983).

Nowadays it is conventional that mixed forests have a higher biodiversity than pure stands. Increased nature conservation value is an added benefit from using mixed forests as a way to control root rot biologically. Stands with alternating rows of conifers and deciduous trees are established at several sites in Lithuania with purpose to address the following question: to plant pure or mixed stands; to thin or not – if we want to avoid root rot attack. Although the cultivation of monocultures is usually the most economic way to produce timber for industrial purposes, the drawbacks of such monocultures have been recognized in many forestry areas and several factors favour the growing of mixed stands. Reduced incidence of *H. annosum* root rot is usually mentioned among these factors (cultivation of allelopathic plants, wider spacing between susceptible trees, higher biodiversity of antagonistic soil microorganisms). It is known that the rate of disease expansion in pines tends to decrease with increasing stand age (Stenlid and Redfern 1998) and this is the main object of the thinning delay.

The present study has been carried out in experimental plantations of *Pinus sylvestris* L. established on the former agricultural land in 1975 in Central Lithuania. In those plantations, thinnings were meant to be delayed for the longest time possible, thus avoiding damage by *H. annosum*. Four different planting schemes have been used (Table 1). Earlier work has shown that some nitrogen-fixing trees (*Robinia pseudoacacia* L., *Amorpha fruticosa* L.) have antagonistic properties to *H. annosum in vitro* (Vasiliauskas et al. 1976, Vasiliauskas 1989). These species were cultivated between rows of pine.

The main aims of this study were to evaluate growth characteristics of experimental Scots pine stands and to search for possible infections by *H. annosum*.

STUDY SITES AND METHODS

Experimental stands of Scots pine

According to programs of afforestation of former agricultural lands in Lithuania, many stands of Scots pine (*Pinus sylvestris* L.) were set on poor and sandy soils. The problem of root rot caused by fungus *Heterobasidion annosum* (Fr.) Bref. arose after the first thinnings. To solve the problem, series of investigations have been done and several recommendations on the setting of resistant stands have been issued (Vasiliauskas et al. 1976). According to these recommendations, the experimental stands of Scots pine (*Pinus sylvestris* L.) have been set in Central Lithuania (Dubrava forest enterprise, Kachergine forest district). Stands of a general area of 6.9 ha have been planted on former arable land, sandy podzol. *P. sylvestris* was mixed with deciduous tree species: *Robinia pseudoacacia* L. and *Amorpha fruticosa* L. European white birch (*Betula pendula* Roth.) was planted in one of four planting schemes and in border-rows between all the schemes. The pure stand of *P. sylvestris* was established as control. The planting schemes are presented in Table 1.

A. fruticosa and *R. pseudoaccacia* have played a role in stand formation for the first 10 years, after they remained just as understorey and degenerated. Now (data from year 2000) there are very few trees of *R. pseudoaccacia* left; *A. fruticosa* looks like small shrubs of low viability. An adjacent older stand of *P. sylvestris* surrounding this experimental is suffering from root rot disease.

Evaluation of tree and stand characteristics

Eleven permanent sample plots (20 x 20 m) were established for the evaluation of tree and stand characteristics: planting scheme I – 5 plots, II – 2 plots, III – 2 plots and IV – 2 plots. Tree characteristics were described by the measurement of certain parameters and visually. Three groups of factors were evaluated: i – factors of productivity, ii – factors of viability and iii – factors of stem quality (Table 1). For the evaluation of growth characteristics in different planting schemes *P. sylvestris* and *B. pendula* trees were involved only. *A. fruticosa* and *R. pseudoaccacia* play only non-significant roles in current stands (as understorey): their evaluation was not made.

Wood sampling and fungal isolations

Sampling of stems was carried out by means of an increment borer as described by Vasiliauskas et al. (1996). Wood samples for the mycological investigations were taken at the root collar (5 cm above ground) of *P. sylvestris* in two sample plots (planting schemes I and II). Nine pines of 75 trees sampled in scheme IV and five pines of 77 trees sampled in scheme II were declining (IV or V Kraft class, transparent crown, short brownish needles). Other pines were sound looking. The increment borer was sterilised in denatured alcohol before each sampling and the bore cores (~4 cm length) were placed into sterile tubes. In a laboratory, the woody pieces were surface-sterilized by flaming for 2-3 s and then placed into the 5 cm diameter Petri dishes containing Hagem agar

(HA) medium: 5 g glucose, 0.5 g NH4NO3, 0.5 g KH2PO4, 0.5 g MgSO4·7H2O, 5 g malt extract, 20 g bacteriological agar and 1000ml H2O at pH 5.5. The Petri dishes were stored at the room temperature for 2 to 4 weeks to grow out fungal mycelia. After, the mycelia (non contaminants) were replaced into new Petri dishes to create pure cultures.

The work on fungal isolation from 152 wood samples was done in purpose to confirm or refute *H. annosum* infection and to study the fungal biodiversity in sound looking and declining pines. Fungal species are being identified by the methodology of DNA sequencing (White et al. 1990) using primers ITS1 and ITS4.

RESULTS

Tree and stand characteristics

Analysis of the field data, collected in 11 sample plots of experimental stands of *P. sylvestris*, showed a negative correlation between the initial (current as well) density of *P. sylvestris* trees and the general productivity (volume of stand per 1 ha) of stands investigated (Table 1). Pure stand of *P. sylvestris* (P, planting scheme IV) had the highest density, but the productivity factors (average volume of stem, volume of stand per 1 ha) were the lowest. Mixed in each second row *P. sylvestris* L. (P)–*A. fruticosa* L. (A) stand provided reverse results (scheme II), showing the highest quality from those points of view. Stands with planting schemes I and III (R-*Robinia pseudoacacia* L., B-*Betula pendula* Roth.) showed intermediate results; however, stand in scheme III occurred to be more productive according to the additional volume of *B. pendula* trees and bigger mean height. Factors of viability corresponded well with factors of productivity, but factors of stem quality showed non-significant differences.

Table 1. The main tree and stand characteristics of the experimental stands planted in 1975 (data from year 2000).

Planting scheme		Initial density of the stand, trees/ha	Factors of productivity					f-rs of viability		f-rs of stem quality	
			Current density of the stand trees/ha	Average diameter of stem at breast height, cm	Average height of trees, m	Average volume of stem, m3	Volume of stand, m3/1ha	Percentage of declining trees in the stand (%)	Kraft class: 1-V cl. 2-IV cl. 3-III cl. 4-II cl. 5-I cl.	Straightness of the stem: 1- v.twisted 2- twisted 3- average 4- straight 5- v.straight	Average thickness of green branches 1- >3cm 2- 2-3 cm 3- <2 cm
PPAPAPAPPR	I	3975	2350	13.1	13.1	0.091	213.9	10.4	4.57	3.96	2.51
PAPA	II	2750	1800	15.2	14.5	0.132	237.6	4.2	4.82	4.19	2.45
PPAPAPAPPB	III	4588	2200	13.5	14.8	0.107	235.7	5.7	4.63	3.92	2.55
PPP (control)	IV	7625	2688	11.3	13.1	0.068	182.8	14.0	4.28	4.11	2.70
Mean		**4735**	**2260**	**13.3**	**13.9**	**0.100**	**217.5**	**8.6**	**4.58**	**4.05**	**2.55**

Mycological analysis

Sixteen different fungal species were isolated from pines in planting scheme IV (control) and 14 species in scheme II. The preliminar results of fungal identification showed that no *Basidiomyetes* have been isolated. Most species were endophytic. Further identification is being continued.

DISCUSSION

It is generally accepted that *H. annosum* enters Scots pine stands mainly through the stumps made during thinnigs (Chase and Ullrich 1983; Swedjemark and Stenlid 1993; Rishbeth 1951). Stands planted on former agricultural land are especially at risk. The planting schemes studied (with exception of control stand: scheme IV) have approximately 2000 trees per 1 ha and the volume of approximately 230 m^3/1 ha. The densities were compared with the density required by the thinning programs currently accepted in Lithuanian forestry (Repsys et al. 1983). It was found out that the stands in schemes I-III do not require thinning. Deciduous tree (shrub) species such as *A. fruticosa* and *R. pseudoaccacia*, planted in alternating rows, have been supressed by pines after a couple of decades, thus providing the acceptable stand density at the age of precommercial thinnings. Our evaluation of tree and stand characteristics showed a significant percent of declining trees in the pure stands of *P. sylvestris* (scheme IV, Table 1), but the results of preliminar mycological analysis did not confirm any infection. The high percentage of declining trees and quite low productivity in this scheme mean that concurency for the space is getting stronger and the stand needs thinning.

The best planting scheme from our point of view is the mixed *P. sylvestris–A. fruticosa* stand (scheme II): there is still little concurrency among the trees that have provided thicker stems. It is obvious that trees that have more space for their growth are producing larger crowns, which makes for worse stem quality (more and thicker branches, longer crown). Our results showed no significant difference among the planting schemes in this criterion. Rows of *B. pendula* in scheme III served as sources of additional volume to the stand when comparing with the similar scheme I. The viability of pines was not affected by the influence of *B. pendula*.

No isolates of *H. annosum* have been found despite the high risk of stands to get infected: an older pine stand surrounding the experimental stands is suffering from this disease. On the other hand, no thinnings were done (no fresh stumps) in the studied stands. Deciduous tree (shrub) species could act as a barrier as well.

CONCLUSION

Stands in the schemes I-III have an acceptable density at the age of 25 and still do not require thinning. No part of the stands showed any signs of root rot attack although some trees were declining due to suppression. *P. sylvestris* trees were inhabited by endophytic fungal species.

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INOCULATION TEST OF *ARMILLARIA MELLEA* ON HINOKI CYPRESS UNDER CONTROLLED TEMPERATURE AND SOIL WATER CONDITIONS

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SUMMARY

Influences of temperatures and soil water conditions on the disease process of Armillaria root rot on Hinoki cypress (*Chamaecyparis obtusa*) were examined with growth chambers and watering computers. Three-year-old seedlings of Hinoki cypress were kept in four chambers controlled as follows: (I) 28°C, watered twice a day, (II) 28°C, watered four times a week, (III) 25°C, watered twice a day, (IV) 25°C, watered four times a week. The seedlings were inoculated with either *A. mellea* or *A. ostoyae*, or left without inoculation for controls. Nine seedlings were used for each treatment. Inoculations were performed in June 2000 and the roots of the seedlings were examined for evidence of infection in March 2001.

A. mellea infected 8-9 seedlings (89-100%) in each chambers. All the infected seedlings that were kept at 28°C and watered twice a day survived. All the infected seedlings that were kept at 25°C and watered four times a week were killed by the fungus. About half of the infected seedlings in each rest chambers survived. The difference of mean radial growth of control seedlings among each group during the year of the experiment suggested that temperatures and soil water conditions influenced on the survival of infected seedlings via host vigor. Inoculation of *A. ostoaye* was unsuccessful because of the death of the fungus in inocula in the early stage of the experiment.

Keywords: *Armillaria mellea*, Hinoki cypress, inoculation, temperature, soil moisture

INTRODUCTION

Hinoki cypress (*Chamaecyparis obtusa* (Sieb. & Zucc.) Endl.) is one of the most important tree species for timber wood in Japan. Armillaria root rot causes mortality on young plantations of Hinoki cypress. The two species, *A. mellea* (Vahl:Fr.) Kummer and *A. ostoyae* (Romag.) Herink are frequently collected from diseased Hinoki cypress (Hasegawa 1994; Ota et al. 1998). These fungi were confirmed to be highly pathogenic on Hinoki cypress by inoculation tests (Hasegawa 1998).

The geographical limitations of the distributions of these two species in Europe are reported to be different from each other: *A. mellea* is found in lower latitude and lower elevation than *A. ostoyae* (Kile et al. 1991). Both two species have been collected from northern and southern area (Hokkaido and Kyushu) in Japan. However, Armillaria root rot in plantations of Hinoki cypress in southern area tends to be caused by *A. mellea* (Hasegawa 1994) and severe incidences of the disease caused by *A. ostoyae* are reported in northern area (Terashita and Sawaguchi 1991). The hypothesis is that the predisposing agents related to climate may be different between these two causal fungi. In this experiment, influences of temperatures and soil water conditions on the disease process of Armillaria root rot on Hinoki cypress were examined using the two fungi.

MATERIALS AND METHODS

Inoculum

Two Japanese isolates of *Armillaria* were used. One is *A. mellea* (AS-1) which was isolated from Hinoki cypress in 1976 in Oita Prefecture, and the other is *A. ostoyae* (93-33) isolated from Japanese red pine (*Pinus densiflora* Zieb. Et Zucc.) in Aomori Pref. Biological species of the isolates were identified by mating tests with European and North American tester strains. Inocula were prepared by placing fragmented mycelia of agar culture of the isolates on autoclaved branch segments of *Quercus serrata* Thunb. (18 cm) with potato dextrose broth and incubated at 25°C for two months.

Preparation, inoculation and growth conditions of seedlings

Seedlings of Hinoki cypress were prepared and inoculated following the modified method of Mallett and Hiratsuka (1988). Two-year-old seedlings individually planted in clay pots were re-planted in larger (18 x 16 cm) clay pots. Together with new soil, two plastic test tubes (1.6 x 10 cm) per one pot were placed around soil fixed by the roots of a seedling. Four months after re-planting, seedlings were put in natural light growth chambers and inoculated. The chambers were controlled as follows: (I) 28°C, watered twice a day, (II) 28°C, watered four times a week, (III) 25°C, watered twice a day, (IV) 25°C, watered four times a week. Watering computers were used for periodical watering. The seedlings were inoculated with either *A. mellea* or *A. ostoyae*, or left without inoculation for controls. Nine seedlings were used for each treatment. Inoculations were performed in June 2000 and the roots of the seedlings were examined for evidence of infection in March 2001. The control seedlings were cut at the ground level and the radial growths of the year of the experiment were measured.

RESULTS

The numbers of infected, not infected, dead and living seedlings of the four chambers are shown in Table 1. *A. mellea* infected most of the inoculated seedlings. The inocula of the fungus had white mycelia under the bark at the end of the experiment period. The numbers of the seedlings survived in spite of infection by the fungus was significantly different among the chambers (Table 2). *A. ostoyae* infection was unsuccessful except for one seedling. In most cases, the inocula of the fungus had no white mycelia under the bark at the end of the experiment period. Death of five seedlings without infection was due to troubles of watering lines.

The mean radial growths of the control seedlings of the four chambers during the year of the experiment are shown in Table 3. The mean radial growth of the seedlings that were kept at 28°C and watered twice a day was better than those of other chambers although only one combination of the values was significantly different statistically.

DISCUSSION

A. mellea was highly infectious under the four different conditions. The result suggested that the fungus adapted wide range of soil water condition (watering four times a week is minimal watering to keep the control seedlings sound) at least at relatively high temperatures such as 25°C and 28°C. On the other hand, resistance of the host to the fungus seemed to depend on temperature and on soil water condition. It can be explained by the fact that temperatures and soil water conditions affect growth of the hosts, and that better growth can make the hosts more resistant by larger amount of photosynthetic products. The control seedlings that were kept at 28°C and watered twice a day showed better radial growth, and all the seedlings inoculated with *A. mellea* under the same condition survived, suggesting that Hinoki cypress could be more resistant under more suitable conditions to grow. Drought may raise mortality of Hinoki cypress infected by this fungus in warmer places in Japan.

The failure of *A. ostoyae* infection could be due to death of the fungus in inocula in the soil before infection. The isolate 93-33 had caused almost the same rate of mortality as AS-1 did to seedlings of Hinoki cypress in previous experiments (Hasegawa 1998). Early death of inocula might by caused by inappropriate preparation of inocula or senescence of the isolate although isolation of AS-1 was much older.

Table 1. The numbers of infected, uninfected, dead and living seedlings under four treatments.

	A. mellea						***A. ostoyae***						**Control**					
	Infected			**Uninfected**			**Infected**			**Uninfected**			**Infected**			**Uninfected**		
Treatment*	**L****	**D**	**T**	**L**	**D**	**T**	**L**	**D**	**T**	**L**	**D**	**T**	**L**	**D**	**T**	**L**	**D**	**T**
I	8	0	8	1	0	1	0	0	0	9	0	9	0	0	0	9	0	9
II	3	5	8	1	0	1	0	0	0	9	0	9	0	0	0	9	0	9
III	5	3	8	1	0	1	0	0	0	8	1	9	0	0	0	8	1	9
IV	0	9	9	0	0	0	0	1	1	6	2	8	0	0	0	8	1	9

* I: 28°C, watered twice a day; II: 28°C, watered four times in a week; III: 25°C, watered twice a day; IV: 25°C, watered four times in a week.

** L: living; D: dead; T: total.

Table 2. Rates of survival of the seedlings infected by *A. mellea* under four treatments.

Treatment*	Rate of survival (%)**
I	100a
II	38bc
III	63ab
IV	0c

* Treatments are explained in the footnotes of Table 1.

** Values followed by the same letter are not significantly different at $\alpha = 0.05$ by Turkey's method of multiple comparison.

Table 3. Mean radial growth of the control seedlings during the year of the experiment under four treatments.

Treatment*	Mean ring-width (mm)**	SD***
I	2.16a	0.57
II	1.45a	0.27
III	1.59	0.56
IV	1.64	0.44

* Treatments are explained in the footnotes of Table 1.

** The two values followed by the letter are significantly different at $\alpha = 0.05$ by Turkey's method of multiple comparison.

*** Standard Deviation.

CONCLUSION

Temperatures and soil water conditions are of critical importance on survival of the Hinoki cypress seedlings infected by *Armillaria mellea*.

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VIRULENCE OF *ARMILLARIA CEPISTIPES* AND *ARMILLARIA OSTOYAE* ISOLATES ON NORWAY SPRUCE SEEDLINGS

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SUMMARY

Preliminary results of a virulence test involving 21 isolates of *A. cepistipes* and 17 isolates of *A. ostoyae* are presented. Two-year-old Norway spruce seedlings of four provenances (two from high and two from low altitude) were planted in plastic pots and inoculated with the selected isolates. After 18 months, seedling mortality caused by *A. ostoyae* was significantly ($P < 0.05$) higher than by *A. cepistipes*. Significant ($P < 0.05$) differences were also observed among isolates of the same *Armillaria* species and among the different spruce provenances.

Keywords: *Armillaria cepistipes*, *Armillaria ostoyae*, Norway spruce seedlings, virulence, provenance

INTRODUCTION

Species of the genus *Armillaria* belong to the most widespread and ecologically significant fungi in natural and planted forests (Shaw and Kile 1991). To date, five annulate *Armillaria* species which differ in geographical distribution, host range and pathogenicity are known in Europe (Guillaumin et al. 1993).

In Switzerland, *A. cepistipes* is the most frequent rhizomorph-producing *Armillaria* species in the soil of mixed mountainous Norway spruce (*Picea abies* (L.) Karst) forests (Rigling et al. 1998). *A. cepistipes* is generally considered a preferentially saprotrophic species with a low virulence (Guillaumin et al. 1993). By contrast, *A. ostoyae* may behave as an aggressive pathogen causing important damages on conifers (Guillaumin et al. 1993; Morrison and Mallett 1996).

The objective of this study was to test the virulence of several Swiss isolates of *A. cepistipes* and *A. ostoyae* on four provenances of Norway spruce seedlings.

MATERIALS AND METHODS

Hosts plants

Virulence of the selected isolates was tested on two years old Norway spruce seedlings from four Swiss provenances: two from low altitude (Bremgarten and Burgdorf: 400 and 600 m a.s.l.) and two from high altitude (Gantrisch and Rüti: 1600 m a.s.l.). In April 1999, the seedlings were planted in 3.5 l plastic pots containing a commercial potting mix (pH 3.7-3.8). Five plants were potted in one pot, four in the margin and one in the center. A section of a plastic pipe (20 cm x 3 cm Ø) was placed near the seedling in the center with care to not wound the roots of the seedling.

Armillaria isolates

Seventeen isolates of *A. ostoyae* and 21 isolates of *A. cepistipes* were tested. These isolates were collected from 1993 to 1998 from 32 different sites in Switzerland where Norway spruce occurs. All *A. cepistipes* and 12 *A. ostoyae* isolates were isolated from rhizomorphs grown in the soil. Five isolates of *A. ostoyae* (C14, C15, C16, C17 and C18) were obtained from mycelial fans in the cambial region of different conifers. The *Armillaria* species were identified in pairings with selected haploid tester strains (Korhonen 1978) of the five European annulate species as described by Harrington et al. (1992).

Inoculum production and seedlings inoculation

Inoculum was produced as described by Rigling et al. (2002). Inoculation was performed at the beginning of May 1999, one month after potting of the seedlings. The plastic pipe was removed and replaced with an *Armillaria* colonized hazelnut segment. Each *Armillaria* isolate was inoculated into five pots (25 plants) containing seedlings from the same provenance. Control seedlings were inoculated with autoclaved, non-colonized hazelnut segments. The pots were randomly placed in the forest nursery of the WSL institute. Watering was assured by an irrigation system with overhead sprinklers.

Virulence of the *Armillaria* isolates

After inoculation, the seedlings were monthly assessed for symptoms of Armillaria root rot (chlorotic foliage and mortality). Seedlings that died were recorded and checked for the presence of *Armillaria* mycelial fans under the bark of the root collar. Dead seedlings were not removed during the experiment. A preliminary analysis of seedlings mortality was conducted 18 months after inoculation (November 2000). Differences in virulence, expressed as the ability to cause mortality, among the *Armillaria* isolates and in susceptibility among the four seedling provenances were analyzed using logistic regression (program DataDesk V. 6.1.1., DataDescription, Inc. Ithaca, NY, USA).

RESULTS

The first seedling with foliar symptoms typical of *Armillaria* infection was observed 11 months after inoculation. Two months later this seedling was dead. Until November 2000 (i.e. 18 months after inoculation), *A. ostoyae* killed 33 of 1700 (1.9%) inoculated seedlings whereas *A. cepistipes* killed five of 2100 (0.2%). All these seedlings showed white mycelial fans under the bark of the root collar. By contrast, no mortality was observed in the control seedlings (Fig. 1). Statistical analysis showed that the seedling mortality caused by *A. ostoyae* was significant ($P < 0.05$) higher than by *A. cepistipes*. However, significant ($P < 0.05$) differences were also detected among isolates of the same species. During the assessment period, 8 of 17 (47%) *A. ostoyae* isolates and 16 of 21 (76%) *A. cepistipes* isolates did not kill any seedling (Fig. 1). In *A. ostoyae*, 75% of the non-virulent isolates were obtained from rhizomorphs grown in the soil whereas the most virulent (C18) was isolated from mycelial fans on an infected tree.

In November 2000, 37 seedlings (2.1%) inoculated with *A. ostoyae* and five seedlings (0.2%) inoculated with *A. cepistipes* were still alive but symptomatic for root disease (Fig. 1). Dying seedlings were observed in pots inoculated with virulent isolates as well as with isolates, which have caused no mortality so far. If we consider both dead and dying seedlings in the analysis, the inter- and intraspecific variations in virulence become more evident.

Seedling mortality also differed significantly ($P < 0.05$) between the low- and high-altitude provenances (Fig. 2). Both *Armillaria* species killed more seedlings from low-altitude provenances (Bremgarten and Burgdorf) than from high-altitude provenances (Rüti and Gantrisch). There were no significant differences in mortality within the same altitude provenances.

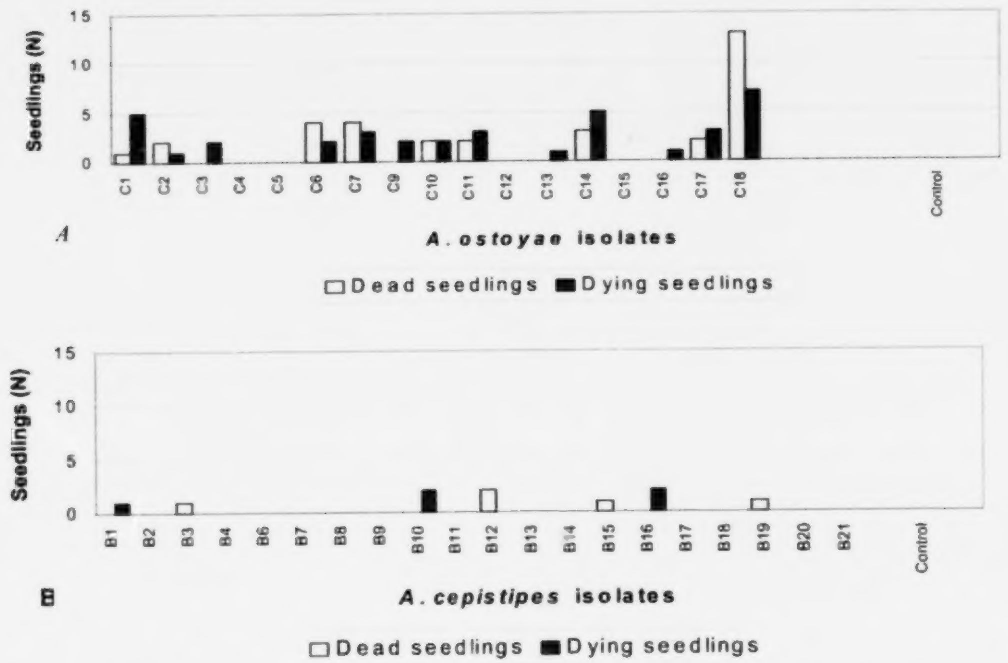

Fig. 1. Mortality (dead and dying seedlings) of Norway spruce seedlings 18 months after inoculation with 17 isolates of *A. ostoyae* (A) and 21 isolates of *A. cepistipes* (B). A total of 100 seedlings were inoculated with each isolate.

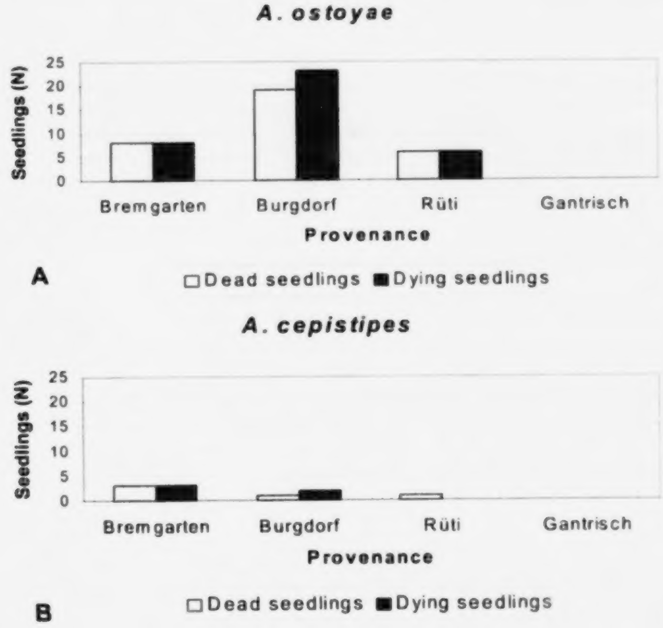

Fig. 2. Mortality (dead and dying seedlings) in the four Norway spruce provenances (Burgdorf and Bremgarten: low-altitude; Rüti and Gan-trisch: high-altitude) 18 months after inoculation with isolates of *A. ostoyae* (A) and *A. cepistipes* (B). A total of 425 seedlings of each provenance were inoculated with *A. ostoyae* and 525 seedlings with *A. cepistipes*.

DISCUSSION

In our inoculation trial, *A. cepistipes* isolates caused about seven fold less mortality than *A. ostoyae* isolates. This result confirms field observations indicating a low virulence of *A. cepistipes* in contrast to *A. ostoyae*, which often acts as a primary pathogen on conifers (Guillaumin et al. 1993). In addition, our study revealed considerable differences in virulence among isolates of the same species. To our knowledge this is the first inoculation study involving a large number of *A. cepistipes* isolates. Intraspecific variations in virulence by *A. ostoyae* were previously reported by Omdal et al. (1995) and Shaw (1977). Our findings support the conclusion of Omdal et al. (1995) that it is indispensable to test a large number of isolates before inferences about pathogenicity and virulence of an *Armillaria* species can be drawn.

The low virulence of several *A. ostoyae* isolates was probably also affected by the time span between inoculation and evaluation (18 months). In a similar experiment, Omdal et al. (1995) began to observe important mortality caused by *A. ostoyae* only 15-18 months after inoculation. It will be interesting to see whether the virulence patterns of our isolates will change during the next year. Low virulence could also be due to a reduced ability of these isolates to produce rhizomorphs under experimental conditions. In fact, some studies suggest a direct correlation between virulence expression and rhizomorphs production (Shaw 1977; Omdal et al. 1995). Since the assessment of the presence or absence of rhizomorphs will be performed only at the end of the experiment, we, at the moment could not evaluate the relation between virulence expression and rhizomorph formation in our trial.

Most of the non-virulent *A. ostoyae* isolates came from soil rhizomorphs whereas the highest mortality was caused by an isolate obtained from an infected spruce. This could suggest that isolates which kill trees in the forest should also be virulent on inoculated seedlings. Nevertheless, two isolates obtained from infected trees also caused no mortality up to now. Shaw and Loopstra (1988) observed that none of the *Armillaria* isolates obtained from dying Alaska-cedars (*Chamaecyparis nootkatensis* (D. Don) Spach) was able to infect seedlings of the same species. Similarly, Mallett and Hiratsuka (1988) observed only a low virulence of isolates of *A. ostoyae* obtained from diseased lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) seedlings under experimental conditions. Thus the behavior of an isolate in nature and in inoculation trials might not always be correlated.

Low-altitude provenances were more susceptible to both *Armillaria* species than high-altitude provenances which could be due to specific differences in susceptibility. Another reason for the higher mortality could be the more vigorous growth of the low-altitude seedlings during the first growing season. As consequence of this growth, roots came faster in contact with the pathogen than the roots of the high-altitude seedlings. Thus, the higher mortality of the low-altitude seedlings is not necessary an indicator of a general lower resistance to *A. cepistipes* and *A. ostoyae*.

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RESROBS: RESISTANCE OF SPRUCE TO ROOT AND BUTT ROT DISEASE, AN EU-FUNDED RESEARCH PROGRAM

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SUMMARY

Spruce breeding programs in several European countries have released clones of *Picea abies* and *P. sitchensis* with superior growth characteristics. The susceptibility of these clones to *Heterobasidion* infection, however, is poorly understood. This research, performed by a consortium of laboratories from EU and EFTA countries, will determine the susceptibility of spruce clones to *H. annosum*, *H. parviporum* and *H. abietinum* in glasshouse and field inoculations, by measuring lesion development. Inheritance of resistance in clones will be determined statistically from these inoculation results. Markers for resistance/susceptibility in clones, allowing predictions of resistance in new crosses, will be determined in parallel work, including qualitative comparisons of resin terpene composition, genetic linkage maps (AFLP, QTL), and the speed and location of biochemical responses of clones (quantitation and localisation of pathogenesis-related proteins). The relative abilities of the clones to wall out infections in the bark and xylem tissues, and the resistance of xylem cell walls to fungal degradation will also be examined. Information from each of these experimental systems will be used to determine suitable biochemical and morphological markers for use in predicting the resistance or susceptibility status of uncharacterised spruce crosses.

Keywords: *Heterobasidion*, spruce, breeding, inoculations, resistance

INTRODUCTION

Currently available control methods for *Heterobasidion* root and butt rot are restricted to stump treatment and stump removal. Stump treatments using either chemicals (Pratt et al. 1998) or the biological control agent *Phlebiopsis gigantea* (Holdenreider and Greig 1998) can give reasonable control of the disease in first rotation plantations. On sites with a long history of forest management, however, where inoculum of *H. annosum* has built up in the woody debris left from previous crops, these control methods are ineffective. Although stump removal can reduce the rate of infection in subsequent crops (Korhonen et al. 1998), it is an expensive process, and use is restricted to sites with light soils and flat topography. Many coniferous forests are located in upland areas, with steep slopes, and have been intensively managed for several rotations, enabling considerable disease build up. The only alternative land use is to plant deciduous trees, which have much longer rotation times and are difficult to grow to high quality standards on sites more suited to conifers.

Within conifer stands badly affected by *Heterobasidion* infection, a small proportion (≥5%) of the host population remains free of infection. Moreover, there is wide variability in the growth rate of the pathogen within naturally infected trees (Delatour et al. 1998). Despite the possibility of random disease escape in these trees, inoculation experiments have indicated that a small proportion of individuals within a population possess genetically determined resistance to *Heterobasidion* infection and/or growth (Delatour et al. 1998). The genetic and physiological basis for this resistance, however, is largely unknown (Karjalainen et al. 1998; Asiegbu et al. 1998).

The rate at which *Heterobasidion* develops in spruce heartwood determines the amount of damage caused within the tree. Work using clones of *P. abies* has shown that disease development is at least partly determined by host tree characteristics (Delatour et al. 1998; Swedjemark et al. 1997, 2001), with strong correlations between infection in 4-year-old plants in the glasshouse and 15-year-old trees in the field. This resistance can be detected using inoculation tests and measuring the extent of lesions developing in the wood and the inner bark (Swedjemark et al. 1997, 2001). Certain chemical properties of the tree, including heartwood pH and terpene profiles, may be correlated with resistance in *P. abies* (Ekman and von Weissenberg 1981). Correlations were, however, low. Factors determining the ability of the bark to resist penetration by the pathogen at root contacts have also been studied (Asiegbu et al. 1998; Solla et al. 2001), and have indicated the importance of host terpenes, stilbenes, lignification and suberisation, and pathogenesis-related proteins.

This paper outlines an EU-funded research programme, recently started, on resistance of *P. abies* and *P. sitchensis* to root and butt rot caused by European species of *Heterobasidion*. Clones of the two host species from full-sib crosses are becoming more widely available for field planting, emphasising the importance of determining the resistance/susceptibility status of selected genotypes to these pathogens. The aim of the project is to exploit natural resistance to *Heterobasidion* species, present in populations of *P. abies* and *P. sitchensis*, the most economically important conifers cultivated in European forests.

METHODS

The most appropriate method to determine the resistance/susceptibility of an individual tree to *Heterobasidion* is through inoculations, followed by estimation of pathogen growth in either the heartwood or sapwood, or by measurement of lesion length in the inner bark (vascular cambium). These techniques, however, are time consuming, and an ability to relate resistance directly to molecular or biochemical traits of the individual tree would greatly increase the efficiency of breeding and selection programmes.

Methods to be used in this project, therefore, include:

- further refinement of inoculations to determine the resistance and susceptibility of individual clones of Norway and Sitka spruce to *Heterobasidion*, using defined isolates of the pathogen;
- elucidating the inheritance of resistance in clones arising from selective breeding programmes, using up-to-date statistical packages;
- developing molecular markers for resistance, based on quality trait linkages (QTLs) and amplified fragment length polymorphisms (AFLPs);
- developing the use of mono- and sesquiterpene analyses as rapid markers for resistance traits;
- elucidating the role of pathogenesis-related proteins in resistance of spruce;
- determining the importance of ligno-suberised boundary zone formation in bark in the resistance of spruce clones to infection by *Heterobasidion* at root-to-root contacts;
- examining the growth of *Heterobasidion* species within the sapwood and heartwood of inoculated clones, in terms of the ability of the pathogens to degrade lignified cell walls, and the hosts' abilities to wall-out (compartmentalise) infections.

CONCLUSION

There is strong evidence for the presence of resistance to infection by, and spread of, *Heterobasidion* in conifers. Moreover, the arguments for using the inherent resistance present in such trees in plantation forestry are compelling, both for reducing the use of undesirable chemicals in forest ecosystems, and for regenerating productive and sustainable conifer plantations on sites with long histories of forest management. Methods allowing the rapid, indirect detection of resistance using molecular and biochemical markers, and an improved knowledge of the host biochemical processes involved in resistance would enable early determination of the susceptibility status of individual clones, prior to extensive propagation for further breeding or outplanting.

The main outputs of the research program will be:

- Quantitative estimation of resistance/susceptibility of available spruce genotypes to European species of *Heterobasidion*.
- Identification of biochemical and genetic markers for early selection of spruce clones showing lower susceptibility to root diseases.
- Greater understanding of the constitutive and induced defences against penetration by and subsequent development of *Heterobasidion* in spruce, and their relationship to resistance/susceptibility.
- The ability to estimate quantitatively the resistance/susceptibility of spruce genotypes to *Heterobasidion* infection and development, based on biochemical and physiological parameters.

These methods will be exploited in the spruce selection and breeding programs ongoing in several EU countries. The main exploitable output of the research, therefore, is the availability of clones of Norway and Sitka spruce with defined resistance to European species of *Heterobasidion*, which may be used in planting programs on badly infested sites or in farm forestry planting, where the site conditions are conducive to disease development.

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VIRULENCE OF *HETEROBASIDION ANNOSUM* S-P HYBRIDS IS DETERMINED BY MITOCHONDRIA

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A potential threat to plants, both in natural and cultivated habitats, is the emergence of hybrid species of fungal phythopathogens with new host specificities or increased virulence. Here we report evidence for mitochondrial control of virulence in hybrid species of *Heterobasidion annosum* (Fr) Bref, causal agent of root and butt rot in conifers. Artificial S-P hybrid heterokaryons, one S-S heterokaryon, and one P-P heterokaryon were produced from four North American homokaryotic *H. annosum* isolates (containing only one nuclear type each). Virulence of the homokaryons and heterokaryons was analysed *in vitro* by daily visual scoring of dead or live pine seedlings for 20 days. The S-P hybrids were characterised by either high or low virulence to pine, characteristic for the P and the S group, respectively. There was a perfect correlation between the mitochondrial type acquired by the hybrids and their virulence. Our results indicate that the mitochondrial genome as such or its interplay with the nuclei play a central role in determining virulence and fitness of plant pathogen hybrids.

ASSESSMENT OF LOBLOLLY PINE DECLINE IN CENTRAL ALABAMA

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Loblolly pine (*Pinus taeda* L.) decline has been prevalent on upland sites of central Alabama since the 1960's. The purpose of this study is to use Forest Health Monitoring (FHM) standards and protocols to evaluate root health of declining trees relative to crown, stem, and site measurements. Thirty-nine 1/6 acre plots were established on loblolly decline sites in nine central Alabama counties. Sites were selected on federal, state, and private industrial lands to measure variables of decline symptoms, age classes and management procedures. Two-root sampling procedures, selective media and soil baiting assay methods were used to isolate pathogenic root fungi. Pitfall traps collected root-feeding insects from which *Leptographium* species have been recovered. Edaphic measurements are ongoing, including soil porosity, bulk density, soil description and nutrient analysis. FHM indicators of tree crown conditions and damages were recorded on all pines in the plots. Preliminary results show a significant correlation with live crown ratio and incidence of *Leptographium* spp. Eighty-four percent of plots recovered *Leptographium* from damaged roots. The pine basal area (ft^2/acre) is significantly reduced with increased incidence of *Phytophthora cinnamomi* Rands. *P. cinnamomi* was recovered from 50% of the plot sites. Histology examination of root damage indicates a significant correlation with reduced growth and root wounding.

ROOT AND BUTT ROT OF *CHAMAECYPARIS OBTUSA* CAUSED BY *PERENNIPORIA SUBACIDA*

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Decay of Hinoki trees (*Chamaecyparis obtusa*) forests was studied in the northern part of Kagawa Prefecture, Japan. Among the 168 Hinoki trees examined, stem rots were found in 33.3, 58.5, and 100% of the trees found in 29, 30, and 34-year-old stands, respectively. Thirteen Hinoki trees were peeled by Sika deer (*Cervus nippon*) and stem rot was found in all of them. All trees with butt rot and 13 trees without it had decayed roots. White mycelia and black flecks sometimes appeared in the decayed wood. Basidiocarps of polyporaceous fungus were often found on felled logs and decayed stumps of *Ch. obtusa* and identified as *Perenniporia subacida*. Basidiomycetous fungus was frequently isolated from decayed wood of roots and stems, and identified as *P. subacida* by comparative study on cultural characteristics. The damage on *Ch. obtusa* by *P. subacida* was first found in Japan. Inoculation experiments proved that the fungus caused the decay of Hinoki trees. White mycelial mats of the fungus were observed to be spreading from old decayed stumps to living neighboring trees through root contact. Paring experiments of isolates showed there existed at least 13 clones of the fungus in this 8900 m^2 site.

A NOVEL METHOD OF COLLECTING AND STORING *HETEROBASIDION ANNOSUM* BASIDIOSPORES FOR USE IN STUMP INOCULATION TRIALS

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Boiled cellophane discs 80 mm diameter were exposed in petri dishes under sporulating *H.annosum* fruit bodies for 24 hours. At the laboratory, the cellophanes were allowed to dry at room temperature for over 8 hours. They were then sorted by the size of the spore deposit, wrapped in aluminum foil, and stored in a domestic fridge (4°C). With this treatment, viability was not affected for several months.

When required for field use, the cellophanes were posted to the experimenter. On the day of inoculation, a spore suspension was made by immersing the cellophane discs in 0.5l water in a wide-mouthed, stoppered plastic bottle which was shaken vigorously. The resulting supernatant was decanted into the inoculation sprayer, and the process repeated a number of times until an adequate volume was achieved. The concentration of viable spores within the inoculum was determined retrospectively by serial dilution of a sample removed from the spray.

PATHOGENICITY OF ROTSTOP TO SITKA SPRUCE IN BRITAIN

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ROTSTOP consists of a genotype of *Phlebiopsis gigantea (Pg)* that decays spruce wood, and it may not occur naturally in Britain. Sitka spruce *(Picea sitchensis)*, which accounts for more than 50% of timber production in Britain, is a native of NW America and has never been exposed to ROTSTOP *Pg*. This work examined the possibility that if used for stump treatment ROTSTOP *Pg* could invade wounds and decay xylem in standing Sitka spruce.

ROTSTOP *Pg* was inoculated directly into decorticated wounds on standing Sitka spruce in Denmark with wood plugs or spores. Its effects on both phloem and xylem were observed after 11-18 months.

15% of ROTSTOP wounds and 11% of sterile wounds showed slight necrosis of phloem, above or below but not to the side of decortications. There was no evidence that necroses were associated with ROTSTOP, and they were most probably caused by tearing phloem tissues while cutting the decortication perimeters. ROTSTOP *Pg* was reisolated from 34 of 132 inoculated wounds, and from two of 132 non-inoculated control wounds.

Columns of stained wood nearly a metre long developed axially around all stem wounds where ROTSTOP *Pg* was inoculated into xylem, and half a metre around control wounds. However, xylem inoculations and control wounds on roots only produced stain columns of 50 cm and (control) 20 cm. Wounds inoculated with spores had fewer and very short stain columns. SEM's of infected wood showed that ROTSTOP *Pg* can cause decay in Sitka spruce (see poster by Bailey, Woodward and Pratt).

This trial indicated that ROTSTOP *Pg* can survive in live Sitka spruce xylem and can decay it, but not to an extent that is a cause for concern.

Incidence and Epidemiology

MONITORING ROOT ROTS IN YOUNG SCOTS PINE PLANTATIONS (UP TO 20 YEARS)

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SUMMARY

In the Zielonka Forest District (central-west Poland), five Scots pine plantations monitored in 1998-2001 for *Armillaria obscura* and *Heterobasidion annosum* disease development proved to be affected mainly by the former pathogen.

Observations of 200 trees per plantation (every spring and fall) showed that there were no significant differences in tree mortality and disease development between three different forest sites (according to Polish forest site typology: fresh coniferous forest, fresh mixed coniferous forest, fresh mixed broadleaved forest). The difference in the disease intensity and development seems to depend on previous stand composition rather than forest site (within the range considered).

Keywords: Scots pine, *Armillaria*, plantation, monitoring

INTRODUCTION

Development of root diseases and tree mortality caused by *Armillaria obscura* and *Heterobasidion annosum* in young Scots pine plantations is essential for their further existence. It should be monitored and forest management approaches should be based on the monitoring results.

Monitoring of *Armillaria* and *Heterobasidion* in Polish forests has been worked out for stands starting from 40 yrs (Sierota and Lech 1996). That is why an approach to plantation monitoring is needed.

The aim of the work was to monitor and evaluate health status and disease development in the Zielonka Forest District (central-west Poland), in which health status of Scots pine plantations has been deteriorating for the past two decades. The plantations investigated (3-15 yrs) were located on three different forest sites (according to Polish forest site typology): fresh coniferous forest (FCF), fresh mixed coniferous forest (FMCF) and fresh mixed broadleaved forest (FMBF). The two former sites are most suitable for Scots pine, the latter is much more fertile and considered very rich for the species. The stand in fresh mixed broadleaved forest contains usually a considerable share of broadleaved trees (mainly oak), which results in many oak stumps after stand removal. Under the circumstances, the new plantation is often exposed to an abundant source of *Armillaria* inoculum. That is why the possible influence of the site on disease intensity was investigated.

MATERIALS AND METHODS

The monitoring method was worked out in the Department of Forest Pathology, University of Agriculture (Manka 1953a, 1953b, 1954; Lakomy and Manka 1998; Manka and Janczyk 1999).

Five Scots pine plantations have been monitored for over 3 yrs (1998-2001) for both diseases. In every plantation, four groups of pines (50 trees each) were observed every spring and fall, and their condition and the disease development was estimated according to a scale (Manka and Janczyk 1999). Age and colour of needles, main shoot increment and symptoms of *Armillaria* and *Heterobasiodion* disease were evaluated, and the trees were given scores - the more affected trees, the higher number of scores. The plantations were situated on the following forest sites (according to Polish forest site typology): fresh coniferous forest (div. 35b), fresh mixed coniferous forest (47k, 85c, 142d) and fresh mixed broadleaved forest (64c).

Isolates of *Armillaria* were tested with the Korhonen (1978) test. For comparison of tree mortality, One-Way Analysis of Variance was applied.

RESULTS

Armillaria root rot was the main disease in the plantations. The species present proved to be *A. obscura*.

The mortality showed no significant dependence on the forest site conditions at $P > 0,05$ (Figs 1, 3, 5, 7 and 9). The plantation on the fresh mixed broadleaved forest site had the greatest percentage of dead trees during the entire investigation period (Fig. 9), but it was not significantly different from those in plantations on both coniferous forest sites (Figs 1, 3, 5 and 7).

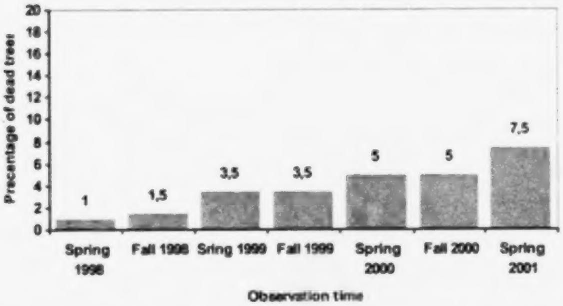

Figure 1. Share (%) of dead trees in observation plots from spring 1998 to spring 2001, division 35b (fresh coniferous forest).

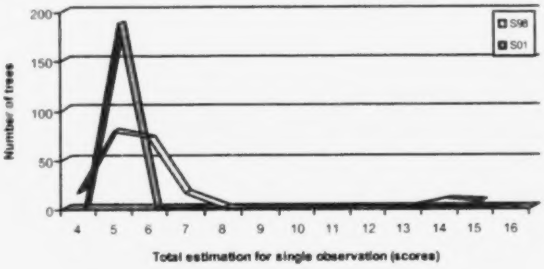

Figure. 2. Variation in health condition of trees in 1998-2001 in division 35b.

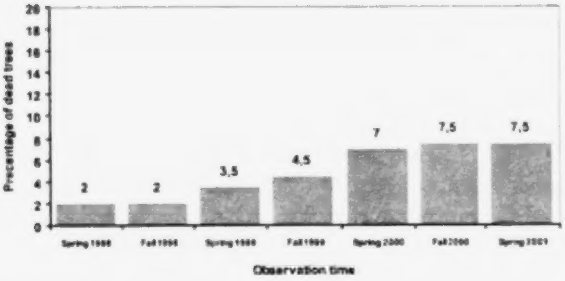

Figure 3. Share (%) of dead trees in observation plots from spring 1998 to spring 2001, division 47k (fresh mixed coniferous forest).

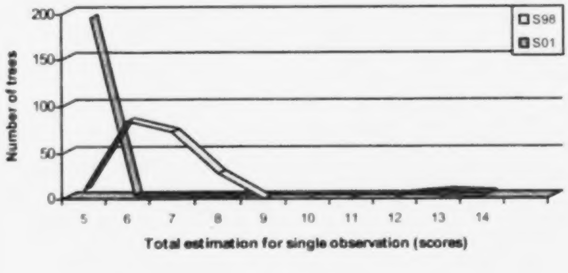

Figure 4. Variation in health condition of trees in 1998-2001 in division 47k.

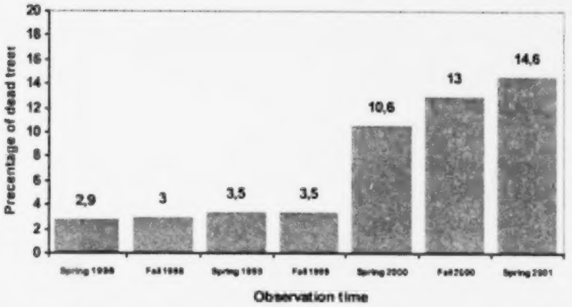

Figure 5. Share (%) of dead trees in observation plots from spring 1998 to spring 2001, division 85c (fresh mixed coniferous forest).

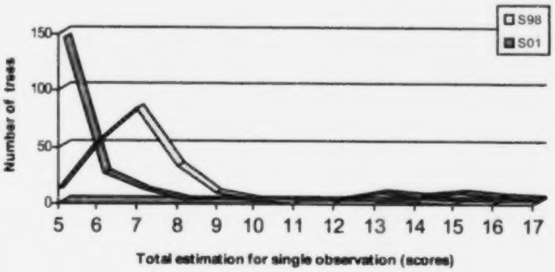

Figure 6. Variation in health condition of trees in 1998-2001 in division 85c.

Change in tree health condition (measured with estimation scores) from spring 1998 to spring 2001 is presented in Figures 2, 4, 6, 8 and 10. In the plantations on all the sites the health status of trees improved, i.e. there were many more pines with 5-7 scores only in 2001 than in 1998. The number of dying and dead trees (14-17 scores) grew considerably only in the fresh mixed broadleaved forest case (Fig. 10).

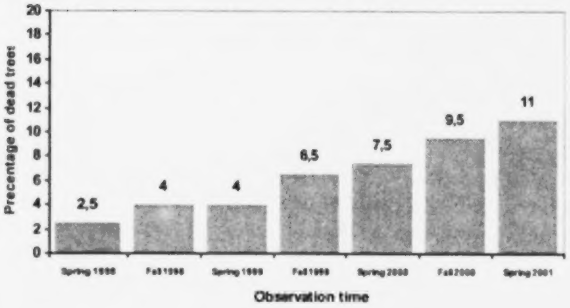

Figure 7. Share (%) of dead trees in observation plots from spring 1998 to spring 2001, division 142d (fresh mixed coniferous forest).

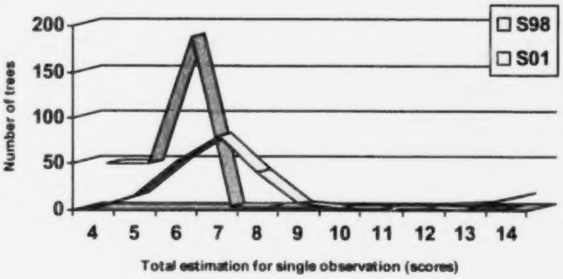

Figure 8. Variation in health condition of trees in 1998-2001 in division 142d.

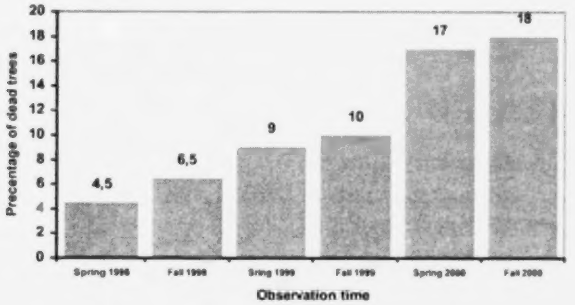

Figure 9. Share (%) of dead trees in observation plots from spring 1998 to spring 2001, division 64c (fresh mixed broadleaved forest).

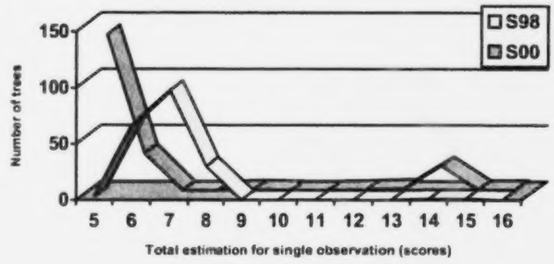

Figure 10. Variation in health condition of trees in 1998-2000 in division 64c.

DISCUSSION

The prevalence of *Armillaria* root rot as the main disease agent in the plantations is similar to the situation in the Olesno Forest District (sout-west Poland, Manka and Janczyk 1999, 2000). The species present there was also *A. obscura*, the most common and severe *Armillaria* species in Poland.

The most fertile site, fresh mixed broadleaved forest, seems more favorable for the *Armillaria* disease development as dead trees are more numerous there (Fig. 9), and the share of diseased and dying pines in spring 2001 is also the greatest (Fig. 10). This may be due to the abundance of deciduous stumps and rich *Armillaria* inoculum, and with weather conditions favorable for *A. obscura*, the spread of the disease may be very quick as it was observed in the hot and wet season of 1998 (Manka and Szewczyk 2000).

Wilting of shoots may be treated as an early symptom of *Armillaria* attack, after which the trees mostly die, as mentioned earlier by Manka (1997, 1998), Lakomy and Manka (1998) and Manka and Janczyk (1999).

CONCLUSION

Armillaria was the main root rot pathogen in the Scots pine plantations investigated.

No significant differences in *Armillaria* disease intensity in plantations on fresh coniferous forest, fresh mixed coniferous forest and fresh mixed broadleaved forest were found.

The spring observation seems to be less important for the monitoring results; the fall observations alone can give proper basis for the monitoring.

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INCIDENCE OF BUTT ROT IN CONSECUTIVE ROTATIONS OF *PICEA ABIES* IN SOUTH-WESTERN SWEDEN

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SUMMARY

The incidence of butt rot at clear felling of 19 mature Norway spruce *Picea abies* (L.) Karst. stands and at first thinning of the subsequent rotations of Norway spruce was examined in south-western Sweden. The incidence of butt rot was estimated on a systematic sample of stumps by visual examination of the stump surfaces shortly after felling. In two stands all stumps were examined at first thinning of the consecutive rotation. The presence of *Heterobasidion annosum* (Fr.) Bref. fruit bodies and *Armillaria* spp. rhizomorphs on stumps of the previous rotation was examined in ten stands. Discs of decayed trees in the present rotation were taken for identification of *H. annosum* infection. There was no relation between the incidence of butt rot at final felling of the previous rotation and the incidence of butt rot at first thinning of the subsequent rotation. *Heterobasidion annosum* was present on old Norway spruce stumps of the previous rotation and the most common cause of decay in the present rotation of Norway spruce. The results suggest that the incidence of butt rot at first thinning of Norway spruce is not necessarily higher on sites where the previous rotation was heavily infected than on sites where the infection in the previous rotation was low.

Keywords: Butt rot, *Heterobasidion annosum*, Norway spruce, *Picea abies*, rotations

INTRODUCTION

Root and butt rot in Norway spruce (*Picea abies* (L.) Karst.) stands, mainly caused by *Heterobasidion annosum* (Fr.) Bref., has resulted in severe economic losses to forestry in Scandinavia (Holmsgaard et al. 1968). The epidemiology of *H. annosum* is a complex process with many factors involved. By basidiospores, *H. annosum* establishes in fresh wounds or on fresh stump surfaces (Rishbeth 1951a, Isomäki and Kallio 1974). The mycelia subsequently spread to healthy trees via root grafts or looser contacts between infected and uninfected roots (Rishbeth 1951a, b). *Heterobasidion annosum* may survive in stumps for over 30 years (Schönhar 1973, Piri 1996). Stenlid (1987) and Piri (1996) have shown transmission of genets of *H. annosum* between consecutive rotations of Norway spruce. The possibility for build up of infection by *H. annosum* in subsequent rotations of Norway spruce has been reported by Jørgensen et al. (1939), Low (1958), Yde-Andersen (1978), and Schönhar (1997). Furthermore, a greater amount of infected stumps originating from the previous rotation will increase the risk for infection in the following rotation (Schönhar 1990).

Since trees with rot in stem, trunk or main roots are more easily wind thrown than healthy trees (Bazzigher and Schmid 1969), the use of Norway spruce has been questioned in areas heavily infected by *H. annosum*. Due to the vegetative growth of *H. annosum* from stumps of the previous rotation the efficacy of stump treatment at thinnings of subsequent Norway spruce rotations has also been questioned (Schönhar 1973, 1990, 1995). However, the build up of infection may be due to both vegetative growth of the fungus from stumps of the previous to the subsequent rotation via root contacts, and a local abundance of sporophores on stumps of the previous rotation that increase the spore infection potential in subsequent rotations (Low 1958, Sinclair 1964).

Before first thinning of a stand, the incidence of butt rot is mainly dependent on the vegetative growth of *H. annosum* from stumps and roots of the previous rotation (Schönhar 1973, 1995). Consequently, the incidence of butt rot at first thinning should be correlated with the incidence of butt rot at final felling of the previous rotation. However, some studies have indicated a lack of correlation between the incidence of butt rot in consecutive rotations (Bornebusch and Holm 1934, Piri 1996, Rönnberg and Jørgensen 2000). Moreover, the numbers of observations were small in some studies and they were carried out outside Sweden.

The aim of this study was to evaluate the relation between the incidence of butt rot at final felling of Norway spruce stands and the incidence of butt rot at first thinning of the subsequent rotation stands of Norway spruce in south-western Sweden.

MATERIALS AND METHODS

The study consisted of 19 sites located in two areas, "Dalby" and Tönnersjöheden ("Tsjö"), in south-western Sweden on former coniferous forest, arable or heath land. The sites were afforested with Norway spruce during the late 19th and early 20th century. The soil textures were sandy loamy till or gravel. The incidence of butt rot was estimated in two consecutive rotations of Norway spruce. The first rotation stands were clear felled during wintertime in the period 1961 to 1981. The time between clear felling and planting of the subsequent rotation varied between zero and three years (Table 1). The number of planted Norway spruce seedlings varied between 1600 and 4500 per hectare (Table 1). At site 13:158 spruce and beech (*Fagus sylvatica* L.) seedlings were strip-wise mixed at planting. Stand 50 was planted at four different spacings. The stand age at first thinning was 24-39 years (Table 1). Site index (Hägglund 1973) was determined for each site as the estimated top height of the stand at a total age of 100 years (Table 1).

The incidence of butt rot after clear felling and first thinning was estimated by visual examination of the stump surfaces shortly after felling. For the sites at "Tsjö" all stumps were observed in five-meter wide strips placed along systematic transects through the stands (Persson 1975). The distance between the transects were adjusted to the area of the site so that approximately 100 stumps were examined within each site. However, the distance between transects was never lower than 25 m. Therefore the number of investigated stumps was lower than 100 in small stands. Due to miscalculations of the number of felled trees, the number of investigated trees was higher than 100 in some cases. At the sites in "Dalby" all stumps within the sites were examined.

As it is difficult to identify standing trees damaged by root and butt rot from external signs (Kallio and Tamminen 1974, Vollbrecht and Agestam 1995), and since there is little or no difference in the incidence of butt rot in different tree classes (Werner 1971, Vollbrecht and Agestam 1995), the butt rot frequency at stump height for the thinned trees within a plot was regarded to be representative of the butt rot frequency in the remaining stand (Holmsgaard et al. 1968, Bryndum 1969, Vollbrecht and Agestam 1995).

Identification of the decay-causing organism was not made at clear felling. However, at first thinning of the subsequent rotation, stumps of the previous rotation was investigated for the presence of *H. annosum* sporophores and *Armillaria* spp. rhizomorphs. In each of the sites within stands 52 and 71 at "Tsjö", ten randomly selected stumps were examined, and at the sites at "Dalby" all old stumps were examined, 92 and 104 stumps for site 1 and 2, respectively. At other sites no examination of old stumps were made. To identify the decay-causing organism at first thinning, stem discs at stump height were taken immediately after felling from 20 randomly selected thinned trees with incipient or advanced decay in each of the sites within stands 13 (except site 161), 52, 71 and 293 at "Tsjö" and all sites at "Dalby". In the other stands thinnings had been carried out 2 - 9 years before this study was carried out. Therefore, to identify the decay-causing organism, bore cores were taken at stump height from 50 randomly selected trees in each stand using an increment borer. In stand 1, no bore cores were taken to avoid conflicts with other scientific activities. Consequently, no identification of the decay-causing organism was made. The bore core was directed towards the pith of the tree (Stenlid and Wästerlund 1986). 2.6-dichlorophenolindophenol was used to make it easier to detect incipient decay (Johansson and Stenlid 1985). If the bore core was found to be decayed, a new bore core was taken. Stem discs and bore cores with advanced or incipient decay were transferred to plastic bags and incubated at +20°C for 10 days. *Heterobasidion annosum* was regarded as the cause of the decay if colonies of *H. annosum* were detected by their conidial stage. Classification of other butt rot causing agents was not made.

Simple linear regression was used to analyse trends in the data material.

RESULTS

There was no relation between the incidence of butt rot at final felling of the previous rotation and at first thinning of the subsequent rotation (β = 0.015, R^2 = 0.0019) (Table 1). There was a slightly positive trend between the incidence of butt rot at first thinning of the present rotation and the total age at first thinning (β = 0.48, R^2 = 0.12). Furthermore, there was no relation between the incidence of butt rot at first thinning in the present rotation and the number of planted seedlings (β = -0.0063, R^2 = 0.2913), the amount of infections caused by *H. annosum* (β = -0.1954, R^2 = 0.020), the site index (β = -0.0093, R^2 = 0.000008), or the time between final felling and planting of the present rotation (β = -0.7707, R^2 = 0.0194).

Heterobasidion annosum was the dominating decay causing fungi at all sites, 50-100%, except for site 13:158, 41%. *Heterobasidion annosum* sporophores on old stumps of the previous rotation were found on four of the ten investigated sites. *Armillaria* spp. rhizomorphs were not found on any of the investigated sites.

Table 1. Incidence of butt rot in two consecutive rotations of Norway spruce. "SI" is the 100-year site index for Norway spruce of the present stand. "Previous" is the incidence of butt rot at final felling of the previous rotation, and "Present" the incidence at assessment of the subsequent rotation. "Delay" is the time between final felling and planting. "Density" is the number of seedlings planted in the present rotation. "Age" is the total age of trees at assessment of the present rotation. "Sporophores" is the frequency of old stumps with fruit bodies of *H. annosum*, and "H.a." is the frequency of infections caused by *H. annosum* at assessment of the present rotation.

Area	Site	SI (m)	Previous (%)	Present (%)	Delay (yrs)	Density (nos.×ha^{-1})	Age (yrs)	Sporophores (nos.)	H.a. (%)
Dalby	1	36	10	38.3	1	2000	28	35	96
Dalby	2	36	75	21.8	1	2000	28	13	92
Tsjö	50 (1)	35	7	18	0	3000[1]	32	-	-
Tsjö	299 (2)	32	15	14	1	3300	26	-	50
Tsjö	292 (3)	33	32	14	0	3300	27	-	87
Tsjö	293 (4)	33	32	10	3	4500	27	-	86
Tsjö	294-295 (5)	33	32	8	0	2500	28	-	73
Tsjö	725 (6)	32	44.8	9.7	0	4500	26	-	86
Tsjö	52;1	30	31.0	16.3	0	3300	36	0	79
Tsjö	52;2	30	27.8	27.5	0	3300	36	10	84
Tsjö	52;3	30	38.3	31	1	2500	35	10	100
Tsjö	71;11	30	71.8	16.2	1	3500	39	0	67
Tsjö	71;12	30	71.8	22.9	1	3500	39	0	88
Tsjö	71;14a	30	45.8	18	1	3500	37	0	64
Tsjö	71;14b	30	52.8	23	1	3700	37	0	77
Tsjö	71;15	28	70.6	18.3	1	3500	37	0	80
Tsjö	13;158	34	33.3	20.6	4	3300	24	-	41
Tsjö	13;159	34	9.2	16.8	4	3300	24	-	63
Tsjö	13;161	34	9.2	8.9	4	3300	24	-	-

[1] Mean value since the number of planted seedlings varied within the site between 1600 and 4500.

DISCUSSION

The most important finding in this study, supported by Bornebusch and Holm (1934), Piri (1996), and Rönnberg and Jørgensen (2000), is the lack of relation between the incidence of butt rot at final felling of the previous rotation and the incidence of butt rot at first thinning of the present rotation (Table 1). Based on logical understanding of the mechanics of disease transmission, there should be a progression in disease severity from one rotation to the next (Schönhar 1990). This should be evident at first thinning. However, several factors may have affected transmission of the disease, such as spore infection of healthy stumps at clear felling, competition by other fungi, stand density, or age at assessment.

It is possible that stumps from healthy trees from the previous rotation may contribute to the transfer of infection of *H. annosum* between consecutive rotations of Norway spruce if they are infected by spores at felling (Bendz-Hellgren and Stenlid 1998, Yde-Andersen 1978). In this study, the estimation of butt rot at final felling was made by visual examination of decayed stumps, and it is possible that the potential infection sources were different from that assessed. Most stands were cut during wintertime when the spore infection risk should be low (Brandtberg et al. 1996), but there were no data on the air temperature during final felling. Temperatures may have been high enough to allow spore infection. Stump treatment at clear felling is normally not carried out in Sweden but could be justified accordingly. This is in contrast with findings of Greig et al. (2001), where stump removal was the only treatment reducing the amount of disease in the consecutive rotation. However, the experiment was not including the use of *Phlebiopsis gigantea* Fr. Jül as an agent for stump treatment.

Coexistence of *H. annosum* and *Armillaria* spp. in stumps has been reported in spruces, firs and pines (Greig 1962). *Armillaria ostoyae* (Romagn.) Herink usually occupies outer tissues at lower parts of the stumps while *H. annosum* occupies inner tissues. *Armillaria ostoyae* thereby diminishes the spread of *H. annosum* to adjacent healthy trees via root contacts (Greig 1962). In this study, however, only *H. annosum* has been identified on stump discs and the presence of *Armillaria* spp. cannot be excluded. Furthermore, the fact that no rhizomorphs of *Armillaria* spp. was found on stumps of the previous rotation does not exclude the possibility that it has been affecting the growth of *H. annosum* between rotations. However, since the previous land-use was coniferous forest, arable or heath lands the probability of presence of *Armillaria* spp. and its importance for the disease transmission should be low (Greig 1962, Rehfuess 1973).

The stands at different sites were not of the same age and since there was a slightly positive trend between the stand age and the incidence of butt rot, it can be argued that the incidence of butt rot at the time for estimation of the present rotation was not comparable between sites. However, if it is assumed that the future development of butt rot in each stand can be based on the incidence of butt rot at the time for assessment in the present rotation it is possible to predict the incidence of butt rot for each stand at 39 years (the oldest site). However, there will still not be any relation between the incidence of butt rot at clear felling of the previous rotation and the incidence of butt rot at first thinning of the present rotation.

According to Stenlid (1987) there is an increasing risk for transfer of infection through root contacts with a decreasing distance between infected stumps and uninfected trees. In this study the number of seedlings in the present rotation did not seem to affect the incidence of butt rot at first thinning. Because *H. annosum* is able to survive in old stumps for many years (Schönhar 1973, Piri 1996), and Norway spruce trees develop extensive root systems, it is possible that the differences in spacing at planting between sites were not large enough and the sample too small for detection of any relations with the incidence of butt rot at first thinning.

CONCLUSION

The results obtained in this study indicate that the incidence of butt rot in consecutive rotations is not related to the incidence of butt rot at final felling of the previous rotation. As supported by e.g. Rönnberg and Jørgensen 2000, stands heavily infected by *H. annosum* at clear felling may be regenerated with a subsequent rotation of Norway spruce without a higher risk for infection until first thinning compared to stands relatively healthy at final felling. Spore infection on stumps healthy at clear felling may contribute to transmission of the disease to the next rotation of Norway spruce, though the efficacy of stump treatment at clear felling can be discussed (Greig et al. 2001). Furthermore, sporophores on old-growth stumps may locally contribute to an increased spore infection potential at thinning of the subsequent Norway spruce rotation, as also reported by Stenlid (1994). Still it seems possible that the economic losses due to *H. annosum* and build up of infection in subsequent rotations of Norway spruce can be reduced by silvicultural practices, i.e. if final felling and thinnings are carried out during winter conditions (Brandtberg et al. 1996) or if stump treatment is carried out at final felling of the old stand and in all thinnings in the subsequent rotation (Korhonen et al. 1994, Brandtberg et al. 1996). However, the importance of spore infection of healthy stumps at clear felling and the mechanics of disease transmission between rotations needs further clarification before stump treatment at clear felling can be economically justified.

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CHARACTERIZATION AND CONFIRMATION OF ENZYME ACTIVITY IN AN ENDOCHITINASE PROTEIN EXPRESSED IN *PHELLINUS WEIRII*-INFECTED DOUGLAS-FIR

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EXTENDED SUMMARY

The fungal pathogen, *Phellinus weirii* Murr. (Gilbn.), causes a serious root disease of Douglas-fir in the coastal forests of western North America (Thies and Sturrock 1995). *Phellinus weirii* has an ectotrophic growth habit (Garrett 1956) that sees it grow along the bark surface of infected roots, eventually penetrating bark and cambial layers to decay wood tissues. Previous biochemical investigations revealed that a 29.3 kDa endochitinase-like protein (ECP) accumulates in *P. weirii*-infected Douglas-fir roots, with concentrations being highest in tissues actually colonised by the fungus.

Plants utilize many defense mechanisms against pathogens including the production of pathogenesis-related (PR) proteins (van Loon 1985). Glucanases and chitinases, belonging to the PR-2 and -3 family of proteins respectively (Hon et al. 1995), have been studied extensively in agronomic pathosystems (Anuratha et al. 1996, Lambais and Mehdy 1996, Recorbet et al. 1998). Chitinase expression occurs in low constitutive levels in most unchallenged plant tissues, however, stresses such as ethylene, mechanical wounding and pathogen or bacterial attack can cause a dramatic elevation in the accumulation of these enzymes (Boller et al. 1983, Kauffmann et al. 1987, Meins and Ahl 1989, Punja and Zhang 1993).

The isolation of functional enzymes for the purpose of activity determination requires the use of non-denaturing agents that do not interfere with enzyme activity. Conifer tissues, which are rich with phenolic substances, inhibit the solubility and electrophoretic mobility of proteins during extraction were it not for denaturing agents such as sodium dodecyl sulphate and 2-mercaptoethanol (Ekramoddoullah 1993, Ekramoddoullah and Davidson 1995). This feature is significantly problematic to researchers attempting to determine the activity of proteins they have isolated from conifers. To enable further studies on the ECP described by Robinson et al. (2000) and its role in the *P. weirii*-Douglas-fir pathosystem, the objectives of this study were to utilize a vacuum infiltration (VI) method to extract the still-active ECP and then confirm its putative chitinase activity.

The VI method effectively extracted intercellular (apoplastic) fluid from needles of *P. weirii*-infected and healthy 9-year-old coastal Douglas-fir. The 29 kDa ECP was the major protein band in the VI extract and older needles had highest concentrations. When SDS-PAGE gels of both needle VI extract and total protein extracts from roots and needles of *P. weirii*-infected trees were Western blotted and then probed with the ECP polyclonal antibody (ECP pab) (Robinson et al. 2000), the ECP pab detected the 29 kDa ECP. These results demonstrate that synthesis of the ECP can also occur systemically in tissues not directly challenged by *P. weirii*. Like many chitinases, the ECP may be both pathogen-induced and developmentally regulated, occurring in all tissues during certain stages of plant growth (Graham and Sticklen 1994). It is likely that the ECP is a multi-purpose enzyme.

By extracting the ECP under non-denaturing conditions we were able to confirm its chitinase activity using a colorimetric assay and a gel overlay technique. Using Western immunoblot and the ECP pab, this latter assay confirmed that the ECP pab recognizes and thus correlates with the chitinase activity of the ECP.

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RISK OF SPREAD OF *HETEROBASIDION ABIETINUM* ON *ABIES ALBA* STANDS IN THE MEDITERRANEAN REGION

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SUMMARY

Recently, the risk of spread of *Heterobasidion abietinum* on Silver-fir in natural forests and plantations in the Mediterranean region and particularly on Italian territory was re-evaluated by analysing and discussing the main epidemic elements related to the host species, the environment and the pathogen. The natural host distribution area (10 *Abies* species are present in the Mediterranean basin) is fragmented in the form of small forests, generally in mountainous sites. In some cases, the populations are genetically differentiated and show different susceptibility to the pathogen. Among environmental and silvicultural factors favouring disease spread, a role may be played by annual precipitation.

INTRODUCTION

In the Mediterranean area, the *Genus Abies* is represented by 10 different species. Some consist of small populations, scattered throughout the Mediterranean basin as a consequence of climatic change during past centuries, but also due to human activities that have reduced available land suitable for growth of *Abies*. In addition, since ancient times the timber of these conifers has been extensively utilised for many purposes, such as construction and ship building (Bernetti 1998; Piussi 1994).

Among Mediterranean firs, *Abies alba* Mill. is one of the most greatly appreciated conifers in this region. Its natural area extends from central Europe to the Mediterranean basin, along the mountain ranges of the Iberian, Italian and Balkan peninsulas. It grows preferably in mixed forests with beech (*Fagus sylvatica*) and other hardwoods (*Quercus, Acer, Ostrya*) (Bernetti 1998).

In Italy, during the middle ages, the nobility and monastic orders owned the majority of Silver-fir forests. The timber of this species was mainly used to build dwellings, castles and churches. Silver-fir cultivation was part of the Benedictine rule, partly for spiritual aims, as the atmosphere in fir stands was held to stimulate meditation, and for economic purposes (Gabbrielli and Settesoldi 1985).

In later history, the management of Silver-fir forests changed from natural regeneration to artificial plantations. However, the changing type of forests and the increasing number of plantations on formerly cultivated agricultural land favoured development of certain diseases, including root rot due to the fungal pathogen *Heterobasidion annosum* F group (= *Heterobasidion abietinum* Niemelä and Korhonen), which causes damage to various *Genera* of conifers, including *Abies, Picea, Pinus* and *Pseudotsuga* (Biraghi 1949; Capretti and Moriondo 1983). Today this disease is endemic in a large proportion of Silver-fir forests including young plantations (1-2 turns) (Capretti 1998; Luisi and Sicoli 1993).

This paper re-examines the risk that damage by *H. abietinum* may worsen in future years and drastically affect *Abies alba* forests growing in a Mediterranean climate.

MATERIAL AND METHODS

Attention focused particularly on 14 sites in the Italian peninsula listed in Table 1. Latitude, annual rainfall, total solar radiation, and type of damage (a-group: dying and b-group: root rot and uprooting of trees) were recorded from each locality. Differences among types of damage inflicted by *H. abietinum* on *Abies alba* stands under various rainfall regimes were evaluated by the x^2 test. The null hypothesis was true for an equal number of infected trees among two classes of annual rainfall (<1000 mm; >1000 mm).

Furthermore, in eight sites among those already considered, the percentage of infected trees was calculated in linear transects (Table 1). The linear correlation between the number of infected trees versus annual rainfall was also calculated.

In each locality investigated, carpophores of the fungus were collected and the isolates tested following the Korhonen method (1978) in order to determine the biological species present in the forests.

RESULTS

The Silver-fir stands considered during this study were located from 44° to 38° of latitude. Annual rainfall ranged from 692 (Gargano) to 2524 (Abetone) mm/year (mean among sites 1294.9 mm); solar radiation (Bartorelli 1967) varied from 2015.5 hn (Normal hours) (Abetone) to 2175.5 (Gambarie) (Table 1). All forests visited were affected by *H. abietinum*; damage was fairly common and geographically distributed, ranging from 56% infected trees at Monte Vulture to 17% at Serra S. Bruno (Fig. 1). The number of infected trees varied according to the amount of annual rainfall. The correlation was significant (r = 0.8602, P > 0.05) (Fig. 1).

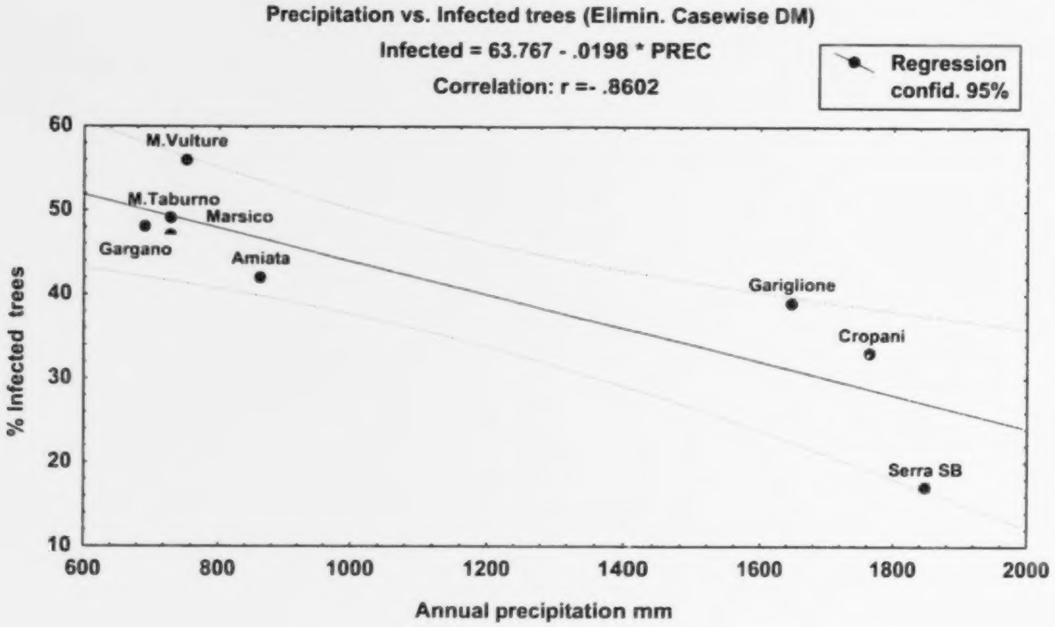

Figure 1. Linear correlation between damage by *Heterobasidion abietinum* and annual precipitation.

Symptoms observed varied from death, root rot to uprooting of trees. Findings also suggested that the type of damage was influenced by total annual rainfall. Thus, tree mortality was most common where rainfall was lower than 1,000 mm per year, and was particularly evident, although with some exceptions, in the south of the Italian peninsula. The x^2 test showed that incidence of infection varied significantly among sites ($x^2 = 10.5$, $P < 0.01$ 1 df).

Solar annual radiation exhibited a minor influence in determining tree death. Generally, Silver-fir mortality was observed at sites where the ratio between rainfall and solar radiation was lower than 0.6 (Table 1).

At the sites examined, *Heterobasidion* carpophores were always abundant on Silver-fir stumps, dead trees and uprooted trees. Interfertility tests following Korhonen (1978) showed that fungal samples collected from the 14 forests all belonged to *H. abietinum* (=*H. annosum* F group).

DISCUSSION AND CONCLUSION

Although *Abies alba* is a typical species of the Mediterranean area, its cultivation is endangered not only by fungal parasites, such as *H. abietinum*, but also by environmental climatic stress such as drought, which is particularly harmful in new plantations. This may partly explain why some of the young Silver-fir plantations least attacked by *Heterobasidion* are found in mountain environments where rainfall exceeds 1600 mm a year, despite being located in the central Mediterranean area (Province of Reggio Calabria).

The foreseeable scenario for the coming years involves progressive warming of the atmosphere, accompanied by a reduction in summer rainfall in certain areas such as the Mediterranean (Le Houerou 1992). If *Heterobasidion abietinum* is present in Silver-fir forests, this could alter environmental conditions in favour of the fungus, by: a) increasing the number of infected trees, especially in areas where rainfall drops below the threshold of 1300 mm/year; b) increasing the death rate when rainfall drops below 1000 mm/year or the ratio between rainfall and annual radiation drops below 0.6.

But it should also be taken into consideration that other factors, such as the planting of pure stands on previously arable land in positions well exposed to sunlight, may contribute to spread of the pathogen and aggravate the damage caused. In severe cases, it may be advisable to exclude Fir completely from new plantations, or even to discontinue cultivation of established fir plantations.

Table 1. The main characteristics of some Silver-fir Italian forests affected by *Heterobasidion abietinum*.

Locality	Latitude	Precipitation mm/year	Radiation hn	Type of damage
Abetone (Pistoia)	44°16'	2524	2015.5	Decay
Camaldoli (Arezzo)	43°48'	1687	2029.5	Decay
Vallombrosa (Firenze)	43°44'	1390	2034.1	Decay
Amiata, Seggiano (Siena)	42°48'	862	2029.5	Mortality
Abeti Soprani	41°52'	945	2084.4	Mortality
Gargano (Foggia)	41°49'	692	2084.4	Mortality
Monte Taburno (Avellino)	41°06'	730	2102.1	Mortality
Monte Vulture	40°57'	752	2106.5	Mortality
Monticchio (Potenza)	40°56'	720	2106.5	Mortality
Marsico Nuovo (Potenza)	40°25'	730	2124.1	Mortality
Gariglione (Reggio Calabria)	39°07'	1646	2154.2	Decay
Cropani Micone (Reggio Calabria)	38°58'	1765	2158.5	Decay
Serra S Bruno (Reggio Calabria)	38°35'	1848	2171.2	Decay
Gambarie (Reggio Calabria)	38°16'	1838	2175.5	Mortality

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INFECTION AND DISTRIBUTION OF *HETEROBASIDION* SPECIES IN STUMPS OF DOUGLAS-FIR

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SUMMARY

Heterobasidion population was investigated in 50 stumps 20 months after the first thinning in a 17-year-old Douglas-fir stand planted after the harvest of a hornbeam coppice mixed with unthinned Norway spruces. No discoloration or decay were present but 19 stumps were infected (38%). *Heterobasidion sp.* was present only in sapwood where it occupied 13% (1 to 64%) of the surface area. 45 roots (20-120 cm long) were collected from nine infected stumps, and 32 roots (70-270 cm) from eight "uninfected" stumps. Forty-one roots were infected in the infected stumps, and two in one 'uninfected' stump. Roots were in general entirely colonised. Partial colonisation was always located next to the stump. 157 isolates of *Heterobasidion* were collected from stumps and roots. The species were identified and somatic compatibility was tested when relevant. Twenty-six sporophores present on stumps of nearby conifer stands were also identified to the species. All the sporophores were *H. abietinum*. *H. abietinum* (F) and *H. annosum* sensu stricto (P) were detected in the stumps and roots. F was more frequent than P (ca. 63% and 35%, respectively) without a significant difference between stumps and roots. *H. parviporum* was detected only in roots (2%). On individual stump sections, all the isolates were from a different SIG. In roots, SIGs were also numerous. Some were present on large root portions (up to 120 cm), and some of them were also present at the stump section. Results suggest that infection was primarily from airborne basidiopores on fresh stumps and infection originating from roots could not be demonstrated. It is confirmed that Douglas-fir stumps may harbour the three European *Heterobasidion* species. Roots were also extensively colonised by *Armillaria* (*A. gallica* and *A. mellea*) which could be a major obstacle to the root transmission of *Heterobasidion*. Moreover, considering that Douglas-fir is a tree species quite tolerant to *Heterobasidion*, it is suggested that the high rate of stump infection observed in that stand will not necessarily result in significant damage.

Keywords: Heterobasidion spp., Douglas-fir, stump, root

INTRODUCTION

Douglas-fir (*Pseudotsuga menziesii*) is a fast growing tree species that was intensively planted in some regions in Europe since the 20th century; it currently produces valuable timber. It became the first conifer planted in France where it covers about 300 000 hectares, the main part of it being in Central France. Douglas-fir suffers from few significant diseases. It may be infected by *Heterobasidion*. However, *Heterobasidion* is far less documented on Douglas-fir as compared to other conifers such as pines, spruces and true firs. A reason for this is that Douglas-fir is considered to have a relatively low susceptibility to that pathogen with only occasional and slight impact. However, foresters are afraid that damage might increase.

The aim of the research was to document natural infection and colonisation of stumps of Douglas-fir by different species of *Heterobasidion*.

MATERIAL AND METHODS

The experimental plot was a Douglas-fir plantation that was 17 years old when the first thinning occurred (October 1997). It is situated in North-East France (forest of Clefmont, 25 km East from Chaumont, Haute-Marne). The soil is loam on limestone; pH value is about 6.0. No sign of infection by *Heterobasidion* was detected in the experimental plot.

History of the plot

Douglas-fir was planted after harvest of the former stand composed of hornbeam coppice mixed with unthinned Norway spruces (30 years old). The site was partially destumped before plantation. No information was available on the *Heterobasidion* situation in that former stand.

Local situation

In the vicinity of the experimental plot, several small conifer plantations were present. Next to the plot was a well growing grand fir plantation (30 years old) severely infected by *Heterobasidion* with symptomatic trees and sporophores abundantly present on stumps; some hundreds of meters apart, sporophores were also detected on a few Douglas-fir stumps in a 30-year-old plantation.

Assessment of stump infection

Twenty months after the first thinning (June 1999), 50 stumps were sampled, randomly distributed over a 100 x 100 m area in the plot. From each stump, a disk 5 cm thick was cut at the ground level. Disks were wrapped individually, then humidified and incubated in the laboratory conditions and examined after about 10 days for presence of *Spiniger meineckellus*. Surface areas of colonisation were estimated on a grid 1 x 1 cm.

Assessment of stump roots colonisation

Ten months later (30 months after thinning; April 2000), nine infected stumps were sampled for roots. Four to six roots were excavated and collected in each, to a length ranging between 0.2 and 1.2 m depending on the possibilities (mean length 0.7 m). Later on (42 months after thinning; April 2001), another set of roots was collected from eight uninfected stumps (four roots per stump; 0.7 to 2.7 m in length; mean length 1.5 m). Disks were cut of from the roots every 10 cm and treated as described for stump disks.

Collection and identification of isolates

Isolates of *Heterobasidion* were obtained in pure culture from sporophores and from the infected disks. Twenty-six sporophores were collected (21 on grand fir and five on Douglas-fir stumps); homocaryotic cultures were obtained from basidiospores. From the infected stumps or roots, 157 isolates were collected under a binocular by picking conidia from the incubated disks (75 isolates from stumps and 82 from roots). In stumps, obtention of one isolate was attempted from each individual infected area. In each root, only two isolates were collected: one from the disk next to the stump (proximal location), and one from the farthest infected disk (distal location). Isolates obtained from conidia were checked for ploidy. Somatic compatibility between isolates ("genets") was examined for all the isolates from each stump, for each pair of isolates from roots, and for a number of isolates from stumps and roots. Specific identification (intersterility groups) was done with the aid of mating tests. Homocaryotic tester strains most often used were the following. ISG-P (*H. annosum*) PA99 (from *Picea abies*, FD de Guéry, Puy-de-Dôme, May 96); ISG-F (*H. abietinum*) PS107 (from *Picea sitchensis*, FD du Mas, Corrèze; Apr. 96). Unambiguous identification was usually obtained using only P and F testers, so, tests with S testers were generally omitted. However, a few isolates that remained ambiguous were submitted to Kari Korhonen.

Armillaria mycelium was occasionally sampled and identified by PCR by Jean-Claude Streito (UMAF, SRPV de Nancy).

RESULTS

Infection of stumps

No discoloration or decay were present on the stump disks collected. Among the 50 stumps investigated, 19 were infected by *Heterobasidion* (38%). Heartwood was quite well developed in the stumps ranging from 16 to 38% of the stump surface area. However, infection never occurred in heartwood but only in sapwood. As shown in table 1, infection of sapwood was very variable for the surface area colonised and for the number of colonies considered independent. It is worth to note that several stumps were intensively colonised (five stumps with more than 24% of the sapwood colonised).

Table 1. Characteristics of the Douglas-fir stumps infected by *Heterobasidion* (19 stumps; mean values).

Surface area		Infection of sapwood	
stump (cm^2)	**sapwood (%)**	**surface area (%)**	**Colonies (no.)**
≈ 500 (diam. 25 cm)	77 (62 - 84)[1]	13 (0.8 - 64)[1]	4.3 (1 – 12)[1]

[1] extreme values in brackets

therwise, presence of *Armillaria* was noted as mycelium fans present beneath the bark of stumps. Among the 50 stumps investigated, 39 (78%) were infected by *Armillaria*. Presence/absence of *Armillaria* and *Heterobasidion* in stumps were not related ($\chi^2 = 1.64$; ddl = 1 ; $\alpha = 0.20$).

Infection of stump roots

All the stumps infected by *Heterobasidion* were also infected to the roots, as shown in table 2. As a rule, all the roots of infected stumps were infected, with few exceptions (two stumps). In contrast, the uninfected stumps were not infected to the roots with only one exception.

Table 2. Distribution of the stumps according to the number of roots infected by *Heterobasidion*.

Infected stumps			Uninfected stumps	
Roots infected (no.)			**Roots infected (no.)**	
3/6	5/6	all (4/4 to 6/6)	None (0/4)	2/4
1 stump	1 stump	7 stumps	7 stumps	1 stump

In all the roots, the infected portions were continuous. In a majority of roots, infection was present up to the last section examined as shown in table 3 ("totally colonised"). Where it was not the case ("partly colonised"), the portion of root infected was always the one next to the stump (proximal portion). No relation was observed between presence of *Heterobasidion* and root diameter; the fungus could have been detected until the small portions of some roots, less than 1 cm in diameter.

Table 3. Length of colonisation of roots infected by *Heterobasidion* (43 infected roots).

	Partly colonised	Totally colonised
No. of roots	15	28
length of root infected (cm)	(10 – 60)[1]	(20 – 120)[1]
diameter of the last section infected (cm)	(8 – 3.5)[1]	(8.6 – 0.5)[1]

[1] extreme values in brackets

Armillaria was present in more roots 42 months after thinning than after 30 months (84% and 67%, respectively; $\chi^2 = 3.26$; ddl = 1; $\alpha < 0.10$). Infection was more developed in roots than in stumps, mycelium being often present in the whole root sections in the roots collected 42 months after thinning. Similarly to *Heterobasidion*, the root portions colonised by *Armillaria* were usually located next to the stumps. However, several cases of discontinuous colonisation were present, with one infected portion located to the medium or the distal part of the root. Presence/absence of *Armillaria* in the roots was not related to that of *Heterobasidion*. In the roots infected by both *Heterobasidion* and *Armillaria*, the two fungi were significantly less frequently associated in a root section than expected (339 root disks; $\chi^2 = 31.09$; dd l= 1 ; $\alpha < 0.001$).

The *Heterobasidion* species

* Sporophores.

All the 26 sporophores belonged to the species *Heterobasidion abietinum* (ISG-F).

* *Heterobasidon* species in stumps and stump roots.

Different situations were present in stump sections and in roots for *Heterobasidion* species and genets, as an example is shown in figure 1.

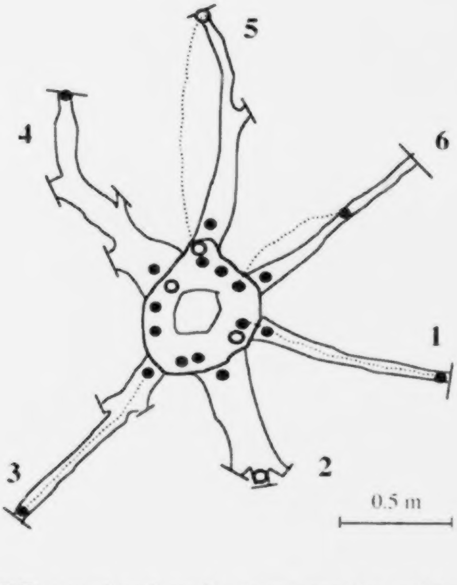

Figure 1. Example of the different situations in stump section and in roots for *Heterobasidion* species and genets (stump # 2; scale is for roots, not for the stump section). Black spots: ISG-F; empty spots: ISG-P; dotted lines connect isolates of the same genet.

The three species *H. abietinum*, *H. annosum*, and *H. parviporum* were detected, as shown in table 4. *H. abietinum* was prevalent in stumps and in roots. Frequency of *H. annosum* was quite high despite no sporophore of that species was found locally. Both species were most often jointly present in individual stumps (see figure 1) where colonies were not significantly different in size (11.2 ± 13.4 and 13.7 ± 22.8 cm^2 respectively). As shown in table 5, with example in figure 1, both species could be present in individual roots, ISGs F and P being randomly distributed (35 roots; $\chi^2 = 3.978$; ddl = 2; $\alpha > 0.10$). *H. parviporum* was infrequent and detected only in roots, however, that species was seldom tested as explained before.

Table 4. Frequency of the *Heterobasidion* species present in naturally infected stumps and roots of Douglas-fir (when several isolates belonged to a same genet they accounted for 1).

	Sporophores	**Stumps**		**Roots**
		all stumps	**(stumps studied for roots)**	
	(26 sporophores)	(19 stumps)	(9 stumps)	(41 roots)
H. abietinum (ISG-F)	100%	63% (47)[1]	55% (30)[1]	62% (40)[1]
H. annosum (ISG-P)	0%	37% (28)[1]	46 (25)[1]	33% (21)[1]
H. parviporum (ISG-S)	0%	0%	0%	5% (3)[1]
TOTAL	100%	100%	100%	100%

[1] number of isolates in brackets

Table 5. Distribution of the roots (no.) infected by *Heterobasidion*, according to the species present at each end.

***Heterobasidion* species**	**F and P**	**F and S**	**P and S**	**F**	**P**	**S**
two different species	10	1	1			
one species				19	6	1

The *Heterobasidion* "genets"

* In stump sections

In the nine infected stumps studied for roots, each isolate collected at the stump section was paired with the others from that stump section. Without exception, all the genets from a stump were all different one from another.

* In stump roots

Pairings between the proximal and distal isolates from the roots showed that different genets could be present in a root. However, in a majority of the roots, the same genet was present at each end of a root (table 6; roots 1 and 3 in figure 1).

Table 6. Distribution of the roots infected by *Heterobasidion* according to the genets present at each end.

***Heterobasidion* species**	**F[1]**	**P**	**S**
two genets (no. roots)	4	3	0
one genet (no. roots)	12	3	1

[1] three isolates were not considered for genet because homocaryotic

* Relation between stumps and roots

All the dicaryotic isolates from roots were paired with the several isolates of the corresponding species obtained from the nearest part of the stump. Among 41 infected roots, only 11 (27%) were found with a genet common to the stump and root (table 7; roots 1, 5 and 6 in figure 1). That situation concerned both *H. abietinum* and *H. annosum*. It is worth to note that in fine roots, a genet located at the proximal end of a root was different of the one present at both the stump and distal end of the root (for example, roots 5 and 6 in figure 1).

Table 7. Genets of *Heterobasidion* present at different locations in stumps and roots (no. of roots involved).

Heterobasidion species	F	P
Location of the genets:		
stump, proximal and distal root	3	2
stump and proximal root	0	1
stump, and distal root	2	3

The *Armillaria* species

Armillaria was identified occasionally (five samples from stumps and 19 from roots). *Armillaria gallica* was found in stumps and roots (five and 12 cases, respectively) and *A. mellea* was found in roots (seven cases).

DISCUSSION AND CONCLUSION

In Douglas-fir stumps (35-70 years old) inoculated with conidia, Cobb and Barber (1968) obtained infection rates of 25% and 38%, with mean values of sapwood colonised of 2% and 35% (inoculated in September and June respectively). Our results show that in younger Douglas-firs (20 years old), *Heterobasidion* may also develop fairly well in the sapwood and in the roots of stumps. Indeed, comparatively, the rate of 38% of naturally infected stumps was quite high, and also the percentage of sapwood colonised in some stumps (24 to 64%) with a mean value of 13%.

The origin of infection in the experimental plot was most likely airborne basidiospores at the time of thinning. The numerous genets present at the stump section and the location of the root portions colonised (proximal portion always infected) are in agreement with that statement. At minimum, it illustrates the classic situation of fresh stump infection. However, infection via roots originating from the spruces of the previous stand cannot be excluded despite the fact that no evidence of it was obtained from the present study.

If the airborne contamination is considered as the only cause of infection, spread of *Heterobasidion* in Douglas-fir stump roots from the stump section would be up to more than 1 m within 30 months (50 cm per year). If we exclude the roots entirely colonised, for which another origin could not be excluded, growth rate would be up to 80 cm in 30 months (30 cm per year). These growth rate estimates are similar to those mentioned in literature for other conifer species (Stenlid and Redfern 1998).

Prevalence of *H. abietinum* in the stumps and roots investigated is another argument for fresh stump infection, as sporophores were abundant and active locally. However, the other species could have been present in the aerial inoculum as it is for *H. annosum* sensu stricto (ISG-P) which was also abundant in the studied stumps and roots. That ubiquitous species is widespread in France and was repeatedly identified in North-East France (Delatour 1998).

Three species of *Heterobasidion* have been detected on Douglas-fir in Europe. However, their relative importance is poorly documented. As tentatively summarised by Korhonen et al. (1998), Douglas-fir is susceptible to *H. annosum* (ISG-P) and occasionally by *H. abietinum* (ISG-F) and *H. parviporum* (ISG-S). In Italy, Capretti et al. (1994) and Sala et al. (1995) stated that *H. abietinum* may be responsible for the death of trees younger than 10 years old when planted in infected *Abies alba* sites. Later on, that species may continue to colonise Douglas-fir stumps without damage on standing trees, and in older Douglas-fir trees (30-60 years old) in pine forest situation, only *H. annosum* sensu stricto was found. In Germany, Siepmann (1988) detected only *H. annosum* sensu stricto in the stem and roots of trees 40-70 years old. In Great Britain, Greig et al. (2001) mentionned that 51% of Douglas-fir (23 years old) may be infected by *H. annosum* sensu stricto; however, no very noticeable decay was detected.

In agreement with these findings, our results show that *H. abietinum* and *H. annosum* may well develop in stumps of Douglas-fir. However, *H. annosum* sensu stricto could be better adapted than *H. abietinum* to colonise Douglas-fir stumps as suggested by its relatively high frequency despite it was probably in the minority in the airborne inoculum.

No information on infection of the standing Douglas-fir trees is available in the studied site, which future is questionable as far as *Heterobasidion* incidence is concerned. The high level of stump infection could suggest a high-risk situation but many factors may influence root transmission of the parasite (Stenlid and Redfern 1998).

In the site investigated, *Armillaria gallica* and *A. mellea* developed extensively in the roots of stumps whether they are infected by *Heterobasidion* or not, but at the root level they excluded themselves from portions of roots. *Armillaria* infection started at the stump collar or at another part of the roots and colonisation of wood developed inward from the bark. That invasion strategy of *Armillaria* must make it difficult in most cases for *Heterobasidion* to reach roots of neighbouring standing trees. Greig (1962) stated that *Armillaria* may limit severely the spread of infection by denying large portions of the root system to *Heterobasidion*.

Otherwise, information collected in France (Delatour 1998) or in other countries in Europe (Siepmann 1988; Sala et al. 1995; Greig et al. 2001), show that Douglas-fir is only occasionally significantly damaged by *Heterobasidion*, illustrating that Douglas-fir is relatively resistant, or tolerant, as compared to other conifer species.

It may be expected that the high infection rate of *Heterobasidion spp.* in stumps observed in this Douglas-fir stand will not necessarily lead to significant incidence of the disease later on.

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INCIDENCE OF ROOT DISEASES IN THE FIR FOREST OF MOUNT PARNIS NATIONAL PARK, GREECE

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SUMMARY

Incidence of root rot pathogens was investigated over an area of 2500 ha of fir forest in the National Park of Mount Parnis. Dead trees and stumps were examined in 20 (0.1 ha) permanent sample plots. *Heterobasidion annosum* (F intersterility group) was widely distributed in the fir forest, occurring in 70% of the plots. *Armillaria mellea* was detected in 25% of the plots, while *Armillaria gallica* was present in 10% of the plots. In 85% of the plots, at least one of the pathogens was found. However, incidence of these fungi was relatively low in the dead trees examined. Upon examination of 329 stumps and trees, which were dead until 1997, *H. annosum* was isolated from 11.55%, *A. mellea* from 1.21% and *A. gallica* from 0.91% of them. From 68 trees that died over a 3-year period (1998-2000) in the plots, 7.35% were found infected by *H. annosum*, 4.41% by *A. mellea* and 1.47% by *A. gallica*. During this 3-year period, 70.59% of the dead trees were observed in the summer and fall of 2000, a year of very limited precipitation. Dead and dying fir trees were found infested by the bark beetles *Phaenops knoteki* and *Pityokteines spinidens*. Another important factor of fir mortality in Mount Parnis National Park is mistletoe (*Viscum album*). Of the trees died over the 4-year period, 85.29% were infected by the mistletoe. The most common saprotrophic fungi detected on fir stumps and dead trees were *Fomitopsis pinicola* and *Trichaptum abietinum* These two fungi seem to play an important role as antagonists of *H. annosum* and *Armillaria* spp.

INTRODUCTION

Mount Parnis is located north to the city of Athens, at a distance of about 30 km (38°10' N). An area of 3800 ha at the high altitudes of the mountain has been designated as National Park since 1961. Most of the area of the Park is covered by fir (*Abies cephalonica* Loud.) at elevations 700-1400 m. Aleppo pine (*Pinus halepensis* Mill.) is the dominant species in lower elevations, forming also mixed stands with fir at elevations 700-1000 m. Soils in the fir-forest are mostly shallow and rocky derived from hard limestone and flysch parent material. The mean annual precipitation at elevations 1020 m has been reported to 750 mm, reaching 1000 mm in certain years, but during dry years this is limited to less than 500 mm (Tsopelas et al. 2001).

Records of fir mortality in Mount Parnis date back to the 1930's. Extensive tree mortality has been reported in certain years in the 1950's and 1960's as well as the 1980's. In all these cases fir mortality has been associated with periodic droughts (Kailidis and Georgevits 1968, Amorgianiotis and Angelopoulos 1996).

Root rot fungi are important pathogens in the fir-forests of Greece. *Heterobasidion annosum* (Fr.) Bref (the F intersterility group), *Armillaria mellea* (Vahl.:Fr.) Kumm. and *Armillaria ostoyae* Velen. have been reported to cause considerable damage in many fir-forests of the country (Tsopelas and Korhonen 1996, Tsopelas 1999). The objectives of this work was to study the incidence of root rot pathogenic fungi in the fir-forest of Mount Parnis National Park and to determine the role of these pathogens in fir-mortality.

MATERIALS AND METHODS

Data were collected from 20 circular sample plots, each 0.1 ha, which were established in 1997 to monitor forest decline. These were systematically located along transects of a 1250 x 1250 m grid, over an area of about 2500 ha of dense fir-forest.

In 1997, all dead trees and stumps in the plots were examined for the presence of root rot fungi. They were included trees that had been dead for about 20 years or less. This was judged by the presence of intact bark in more than half of their circumference at the root collar (Filip and Goheen 1982). Also, they were examined all the trees that died in the following 3 years (1998, 1999, 2000). At least two roots, on opposite sides of trees and stumps, were excavated and examined for sings of root rots. A section 10-15 cm from the roots was cut, placed in a plastic bag and transferred to the laboratory. Following root excavation each tree was felled and trunk disks were cut at ground level and were also transferred to the laboratory.

The presence of *H. annosum* in trees and stumps was detected from the characteristic white, stringy decay in the roots and the trunk of felled trees. Hollow stumps were also examined for the presence of basidiocarps of *H. annosum*. The roots and the root collar were inspected for the presence of the characteristic mycelial mats of *Armillaria* species and the occurrence of rhizomorphs on the roots (Filip and Goheen 1982, Tsopelas 1999). During the autumn the plots were inspected for the presence of basidiocarps of *Armillaria* spp.; they were collected basidiocarps from the sample plots as well as from other areas of the fir-forest. Also, basidiocarps of other wood rotting basidiomycetes were noted and collected for identification from the sample plots.

Isolations from root samples and trunk disks were carried out without surface disinfection. The samples were washed well in tap water and dried. The isolation medium was potato dextrose agar (PDA) amended with fungicides and antibiotics (Tsopelas and Korhonen 1996). Also, isolations from basidiocarps of *H. annosum* and *Armillaria* were performed on the same type of medium. Only heterokaryotic cultures were obtained in this study. Pure cultures were maintained on 2% w/v malt extract agar (MEA). This substrate, along with carrot agar, were also used for identification of *Armillaria* species, on the basis of morphological characteristics of the vegetative mycelium (Guillaumin et al. 1989, Tsopelas 1999). However, in most cases, identification of *Armillaria* species was confirmed in pairings on 2% MEA of the isolates (putatively diploid) with haploid testers (Korhonen 1978a, Guillaumin et al. 1991). These were belonging to the three *Armillaria* species that occur in southern Greece: *A. mellea*, *A. gallica* and *A. tabescens* (Tsopelas 1999). The intersterility group of the isolates of *H. annosum* were determined in pairings with homokaryotic tester strains F, P and S, on 1% MEA (Korhonen 1978b, Tsopelas and Korhonen 1996). In certain plots, when *H. annosum* was isolated from neighboring trees and stumps, pairings were carried out between these isolates to determine the vegetative incompatibility groups (VIG's) and study the spread of the fungus between trees (Korhonen 1978b).

RESULTS AND DISCUSSION

Three species of root pathogenic fungi were identified in the fir-forest of Mount Parnis: *Heterobasidion annosum* (the F intersterility group), *Armillaria mellea* and *Armillaria gallica* Marx. & Rom. In 17 of the 20 sample plots (85%) at least one of these fungi was present. *H. annosum* was found with the highest frequency; it was recorded in 14 (70%) of the plots. *A. mellea* was present in five (25%) of the plots and *A. gallica* was detected in two (10%) plots. In two of the plots, *H. annosum* and *A. mellea* occurred together; in one plot *A. mellea* and *A. gallica* were found together and in another plot *H. annosum* and *A. gallica* occurred together (both were found on the same tree which was dead for many years). Although these fungi were well distributed over the entire area of the fir-forest, the incidence of infection was relatively low. Upon examination of 329 stumps and trees which were dead until 1997 (approximately the last 20 years), *H. annosum* was isolated from 11.55%, *A. mellea* from 1.21% and *A. gallica* from 0.91% of them. From 68 trees that died in the following 3 years (1998-2000), 7.35% were found infected by *H. annosum*, 4.41% by A. mellea and 1.47% by *A. gallica* (Table 1). However, very often the roots of fir trees were growing in fissures between rocks of limestone and only a small part of the root system was excavated. Therefore, it is possible that some of the roots of the dead trees were infected by these fungi and were not detected in our investigation.

In two of the plots, *H. annosum* was detected only in a single tree. In the rest (12) of the plots, the number of trees and stumps, which were infected by *H. annosum*, ranged from two to nine. In nine of the plots they were found genotypes (VIG's) of *H. annosum* that had spread secondarily to more than one tree or stump, two to four genotypes were determined in each plot. The longest distance that a single genotype was present in all sites was 7 m. However, very often adjacent trees or even the same tree were infected by different genotypes of the fungus. *A. mellea* was mostly found singly in the plots, except in one case that the fungus was isolated from three adjacent trees. This fungus though has been observed in many areas of Mount Parnis to cause mortality of fir-trees in groups of two to seven trees. *A. gallica* was found in one of the plots that have infected two small fir trees suppressed in the understorey, while in the other plots its presence was saprotrophic.

Table 1. Incidence of root pathogens in sample plots

Fungal species	**Percent of infection (%)**	
	Stumps and trees dead until 1997[1]	**Trees that died from 1998 to 2000**[2]
Heterobasidion annosum	11.55 (38)[3]	7.35 (5)[3]
Armillaria mellea	1.21 (4)	4.41 (3)
Armillaria gallica	0.91 (3)	1.47 (1)

[1] Total number of trees and stumps 329, [2] Total number of trees 68, [3] Number of trees and stumps infected

H. annosum was isolated from trees that had been dead for several years as well as from recently dead trees, inside and outside the plots. In old stumps and trees that had been dead for many years, *H. annosum* was detected in decayed wood below the ground line, while other types of decay were present above the ground line. Similar observations have been reported by Filip et al. (1992) in fir-tree stumps in forest of western United States. According to these authors, the presence of viable *H. annosum* below ground with other types of rot above ground is an indication that the roots were infected live and further colonization of the root system occurred after the death of the tree. Infections of *H. annosum* in root system of living fir-trees may be common in Mount Parnis; in a few cases, the fungus was isolated from wood cores taken from the base of the trunk of live trees. This is further supported by the presence of recently windthrown trees with extensive root infection, in which the crown was still green without any obvious symptoms of disease.

A. mellea was detected in recently dead fir-trees by the presence of mycelial mats in the cambial zone of roots and the root collar, often extending in the above ground portion of the trunk up to 2.5 m in height. However, in trees which had been dead for several years, mycelial mats could not be found in the above ground portion of the trunk or even the root collar; only in roots below ground could be detected. Rhizomorphs of *A. mellea* were not very frequent in Mount Parnis. More often they were detected rhizomorphs of *A. gallica* (mostly outside the plots) in old stumps.

Basidiocarps of *H. annosum* were mostly found inside the hollow stumps, where moisture conditions were favourable for their development. Very rarely basidiocarps of this fungus were observed around the root collar of trees at the ground level, usually covered by the litter. Fruitings of *Armillaria mellea* were observed in many areas of the fir-forest of Mount Parnis, during the years of observation. They appeared usually in October and were associated with dead trees and stumps; in a few cases, they were also associated with live trees, in which, only a part of the root system and root collar was infected. Basidiocarps of *A. gallica* were relatively rare during the four years of observation. However, they had been recorded in the fir forest a year earlier (1996) in abundance. They were mainly fruiting on stumps but also on bare ground, connected though to wood material with rhizomorphs.

Fir mortality was 0.96% in 1998 and 0.28% in 1999, but it was significantly increased in the year 2000 to 3.65%. This is interpreted to 7.0 trees/ha in 1998, 2.0 trees/ha in 1999 and 26.5 trees/ha in 2000. In the year 2000, in which high tree mortality was recorded, precipitation was significantly reduced. Although we do not have information from Mount Parnis data from other areas of the Attika region show that precipitation during the hydrologic year 1999-2000 was reduced by more than 50%. High fir mortality has been observed in many areas of Greece during periods of extensive droughts (Kailidis and Georgevits 1968, Tsopelas et al. 2001) Generally, tree mortality was more severe in southern and eastern facing slopes, in sites of low productivity. In certain areas of Mount Parnis, fir grows under adverse site conditions, in rocky shallow soils. Fir mortality has been severe in these areas over the years, especially near the margins of the fir range, at elevations 800-1000 m. As a result, Aleppo pine is taking over in certain sites where fir was growing a few decades ago (Tsopelas et al. 2001). Root pathogenic fungi were widely distributed in the fir forest and they certainly play a role in tree mortality of many trees. However, the majority the fir-trees that die every year in Mount Parnis do not seem to be affected by these pathogens.

Dead and dying fir-trees were found infested by bark beetles, whether they were infected by root rot fungi or not. The most common bark beetle was *Phaenops knoteki* Reitt. (Buprestidae), this was observed in almost all of the dead trees. Also, a considerable number of the dead trees were infested by *Pityokteines spinidens* Reitt. (Scolytidae). Bark beetles play an important role in fir mortality. They infest and kill trees suffering from water deficiency and/or affected by other abiotic and biotic factors (Ferell and Hall 1975). Among the biotic factors are root pathogenic fungi. Another important biotic factor that affects tree vigor and mortality in Mount Parnis is the mistletoe (*Viscum album* L.). Of the trees that died over the 3-year period, 85.29% were infected by mistletoe; a significant proportion of these trees were heavily infested by this parasite.

The most common wood decay fungi in the fir forest of Mount Parnis were *Trichaptum abietinum* (Fr.) Ryv. and *Fomitopsis pinicola* (Fr.) Karst. *T. abietinum* was identified by its basiocarps in all of the sample plots, occurring in stumps and dead logs, occasionally it was also found on the dead portion of the trunk of living firs. *F. pinicola* was detected in 13 of the plots (65%), but it may be underestimated. Basidiocarps of *F. pinicola* were not very frequent; the fungus was isolated from stumps and dead trunks, causing characteristic brown rot. These two decay fungi seem to play an important role as antagonists of root pathogenic fungi. They were observed quite often on trees and stumps also infected by *H. annosum* and *A. mellea*. Other decay fungi identified on fir stumps and dead trunks were: *Pleurotus ostreatus* (Jacq.:Fr) Kumm., *Ischnoderma benzoinum* (Wahl:Fr.) Karst., *Ganoderma carnosum* Pat., and *Pholiota aurivella* (Batsch:Fr.) Kumm.

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ROOT AND BUTT ROTS IN SEMI-MATURE, PRE-COMMERCIALLY THINNED STANDS OF BALSAM FIR IN NEWFOUNDLAND

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INTRODUCTION

Root and butt rots are the single most important pest affecting overall average growth and yield in Boreal spruce-balsam fir forests. They are considered "The Hidden Enemy" (Whitney 1988) as the majority of activity by their causal agents, decay fungi, occurs underground unnoticed. Root and butt rot fungi kill small, lateral roots and decay structural heartwood of the major roots and butt section of living trees. The result, wood volume losses due to reduced growth, tree mortality, windthrow and scaled butt cull. An extensive study in naturally regenerated stands of spruce and balsam fir in Ontario (Whitney 1989) revealed root rot damage was highest in balsam fir, intermediate in black spruce and least in white spruce.

In the mid 1970s, a pre-commercial thinning (PCT) program started in Newfoundland. to release overstocked balsam fir stands, based on the expectation of significant gains in piece size and merchantable volume at a reduced rotation age. Today PCT, to about 2000 stems/ha, is one of the major silvicultural treatments used on second growth spruce-fir sites. Post cut assessment in 1996 of commercial thinning (CT) trials in central Newfoundland (some of the oldest PCT balsam fir stands, 35-40 yr age) revealed 26% of the cut stumps had ground level decay (GLD) (Fig. 1a). High levels of GLD after CT have been reported from PCT balsam fir stands on the Northern and Avalon Peninsulas where considerable windthrow in certain areas has also been observed (Fig. 1b). In 1997 a cooperative study between the Newfoundland Department of. Forest Resources and Agrifoods and the Atlantic Forestry Centre (Newfoundland) was initiated to survey butt rot in semi-mature PCT balsam fir stands. It was not until 2001 that an opportunity was available to assess and compare butt rot development in similar unthinned stands. Preliminary results of these surveys are presented.

PROJECT OBJECTIVES

Based upon preliminary results from the PCT and unthinned surveys a proposal is being developed to address the following objectives:

(1) To assess the impact silvicultural treatments, specifically PCT, have upon root rot development
(2) To isolate and identify the major root rot fungi
(3) To determine the variability of root rot by host species as affected by forest site type
(4) To develop hazard rating systems and treatment options to effectively manage for root rot
(5) To determine the incidence and severity of root rot in natural and treated spruce-fir stand

METHODS

Temporary 0.01 ha circular plots were the standard sample plot size. For the 1997 survey, balsam fir stands thinned for a minimum of 14 yr were selected in three Forest Management Districts. Each crop tree was measured for height, diameter, crown size and canopy position. Two increment cores, taken at right angles at ground line were used to determine the presence or absence of butt rot. Trees with any evidence of rot were felled and dissected according to procedure by Laflamme et al. (1977) to obtain rot volumes and samples for decay fungi isolation. A minimum 20% of crop trees per plot were sampled if butt rot was not detected by coring. For the 2001 survey, plots were located in unthinned stands adjacent to or similar forest site types within close proximity of PCT plots previously sampled. For the unthinned plots all trees above 7.0 cm DBH were measured and felled. Trees with butt rot were dissected to obtain rot volumes and samples as described above.

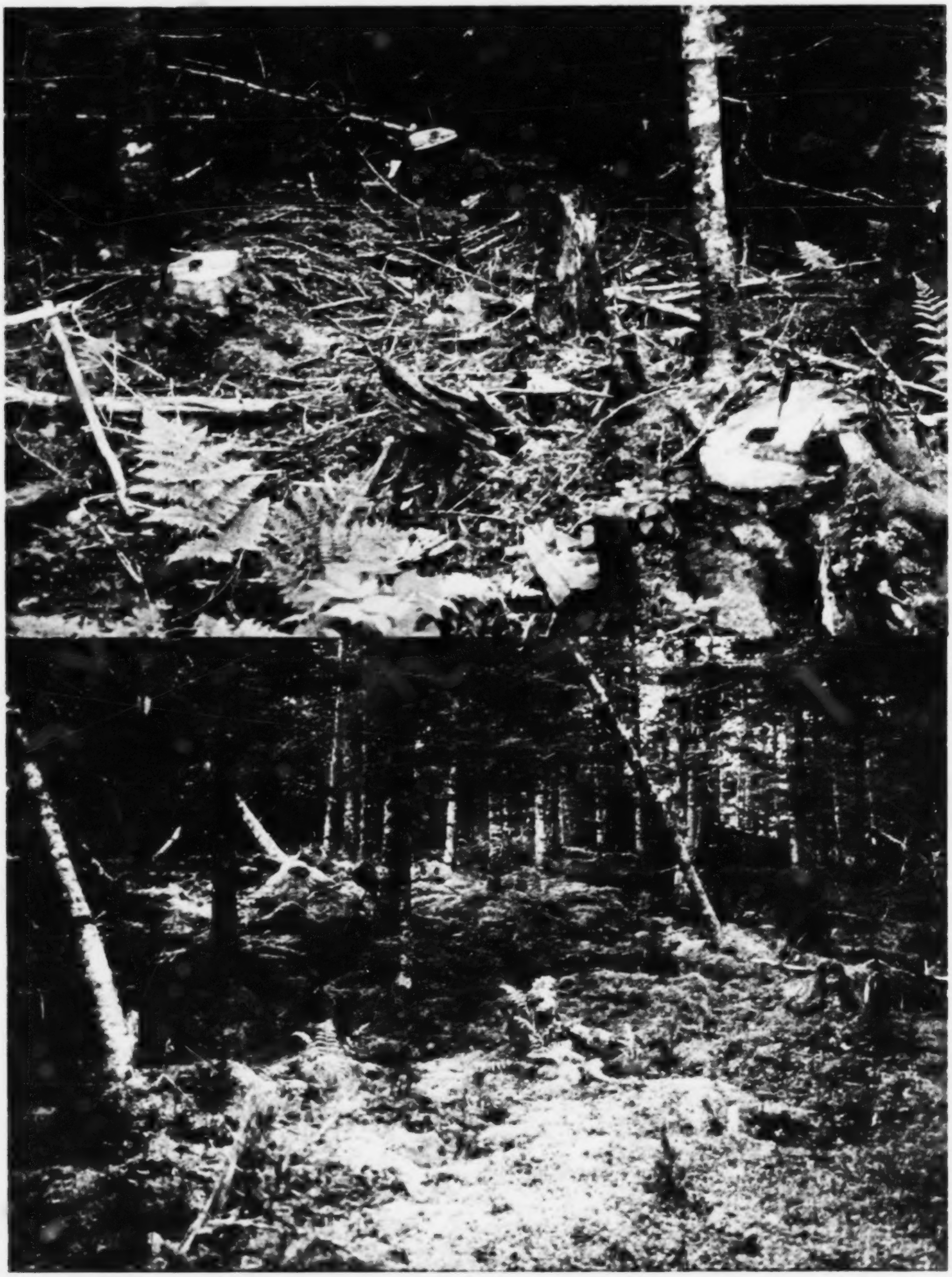

Figure 1. Root rot damage in pre-commercially thinned balsam fir stands resulting in (a) ground level decay and butt cull in the central region and (b) mortality and windthrow in the Avalon region of Newfoundland.

RESULTS AND DISCUSSION

Thirty eight plots were sampled in the 1997 butt rot survey of semi-mature PCT balsam fir stands. Average age of the stands was 33 yr, average time since treatment was 16 yr and average stand density was 2003 stems/ha. Of the balsam fir crop trees, 18.7% had some degree of butt rot accounting for 0.25% of the total balsam fir volume. Fig. 2a and 3a indicate a very low volume of rot as percentage of total tree volume. Volume of rot at this age is relatively unimportant compared to the percentage of stems with butt defect and the rate of increase as stand age increases (Fig. 2b) and years since PCT treatment (Fig. 3b). These results warranted establishing the comparison plots in unthinned stands.

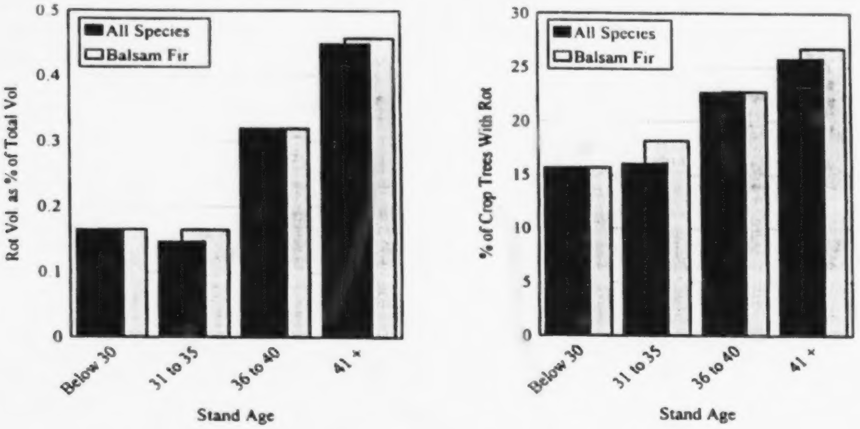

Figure 2. The percentage tree volume (a) and percentage of trees affected (b) by root rot in pre-commercially thinned stands as represented by stand age (English and Warren 1999).

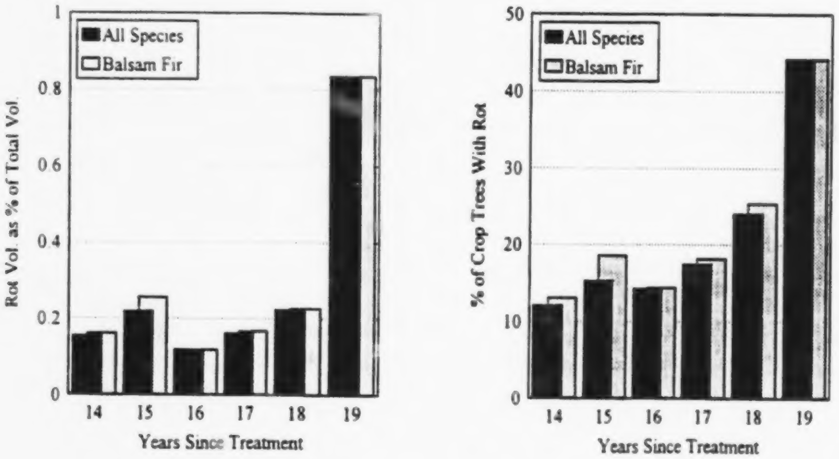

Figure 3. The percentage tree volume (a) and percentage of trees affected (b) by root rot in pre-commercially thinned stands as represented by years since treatment (English and Warren 1999).

In 2001, six unthinned plots were established in two areas in close proximity of PCT plots, three in each of the Gull Pond and Goose Arm areas. Average stand age of the plots in the Gull Pond area were similar taking into account the time between sampling (PCT 33 yr in 1997 vs unthinned 37 yr in 2001). In the Goose Arm area the unthinned plots at 45 yr in 2001 were respectively older than PCT plots at 32 yr in 1997.

Comparisons of PCT vs unthinned plots revealed significantly higher percentage of rot volumes (Fig. 4a) and percentage of stems infected (Fig. 5a) in the PCT plots. Even when considering the conservative estimate of rot in PCT plots (detected only by coring), the percentage of rot volume there was 7 to 26 times greater and the percentage of stems infected was 2 times greater in the PCT plots compared to unthinned plots.

Impacts of these differences are all the more important when considering that in the PCT plots there is, on average, 55% less total bF volume (Fig. 4b) and 75% fewer stems/ha (Fig. 5b). In the Ontario survey of natural regenerated balsam fir stands (ave. age 70 yr), tree mortality of 16% and scaled butt cull of 17% resulted in a 33% total wood volume loss to root and butt rots when the actual wood volume loss to advanced butt decay was only 1.5% of the gross tree volume (Whitney 1989).

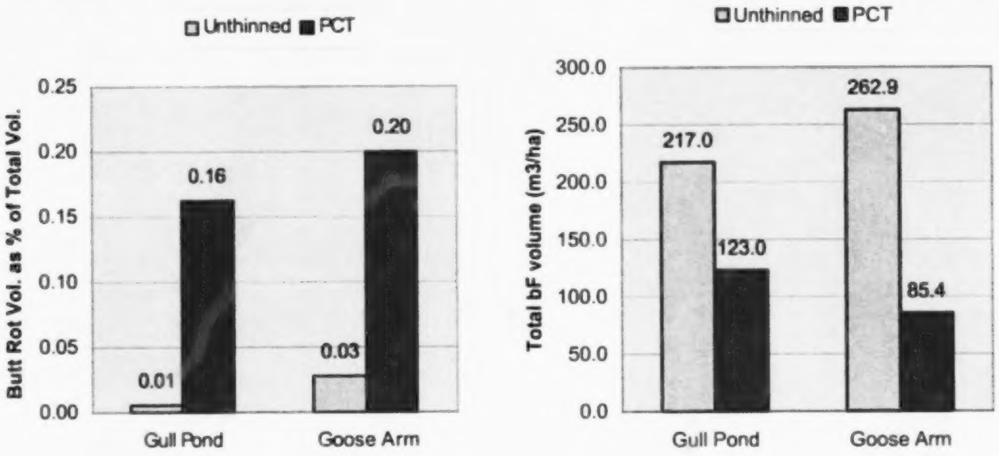

Figure 4. Percentage of butt rot volume in unthinned and PCT plots (a), compared to the total balsam fir volume (b) for the Gull Pond and Goose Arm areas.

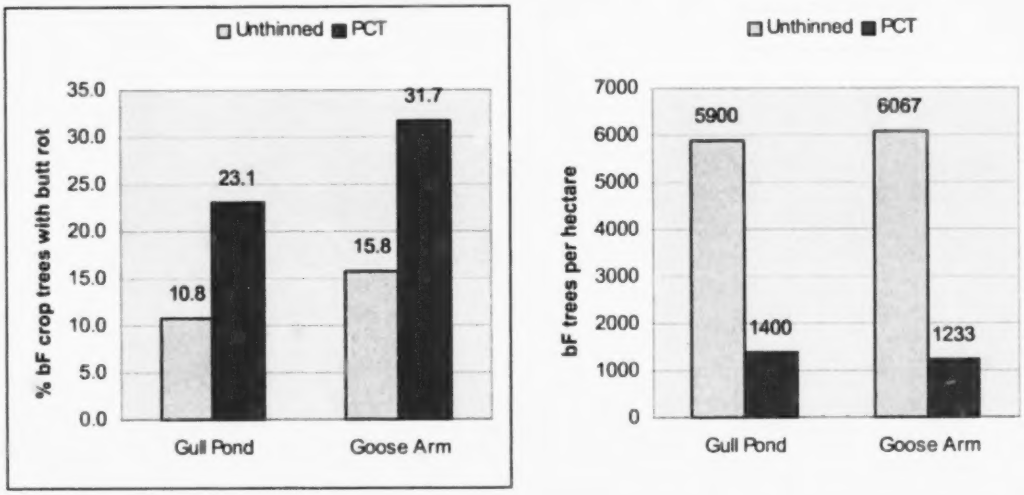

Figure 5. Percentage of crop trees with butt rot in unthinned and PCT plots (a), compared to the total number of balsam fir trees (b) for the Gull Pond and Goose Arm areas.

Root wounding and breakage caused by increased stem sway (Rizzo and Harrington 1988) are suspected as the major cause for increased incidence of root and butt rots in PCT balsam fir stands. PCT to about 2000 stems/ha at the prescribed times of 10-15 yr age and 2-3 m in height (exhibiting crown dominance) results in abnormal crowns to stem and root support (Fig. 6). Many regions of Newfoundland are exposed to high winds which contribute to excessive stem sway under these conditions.

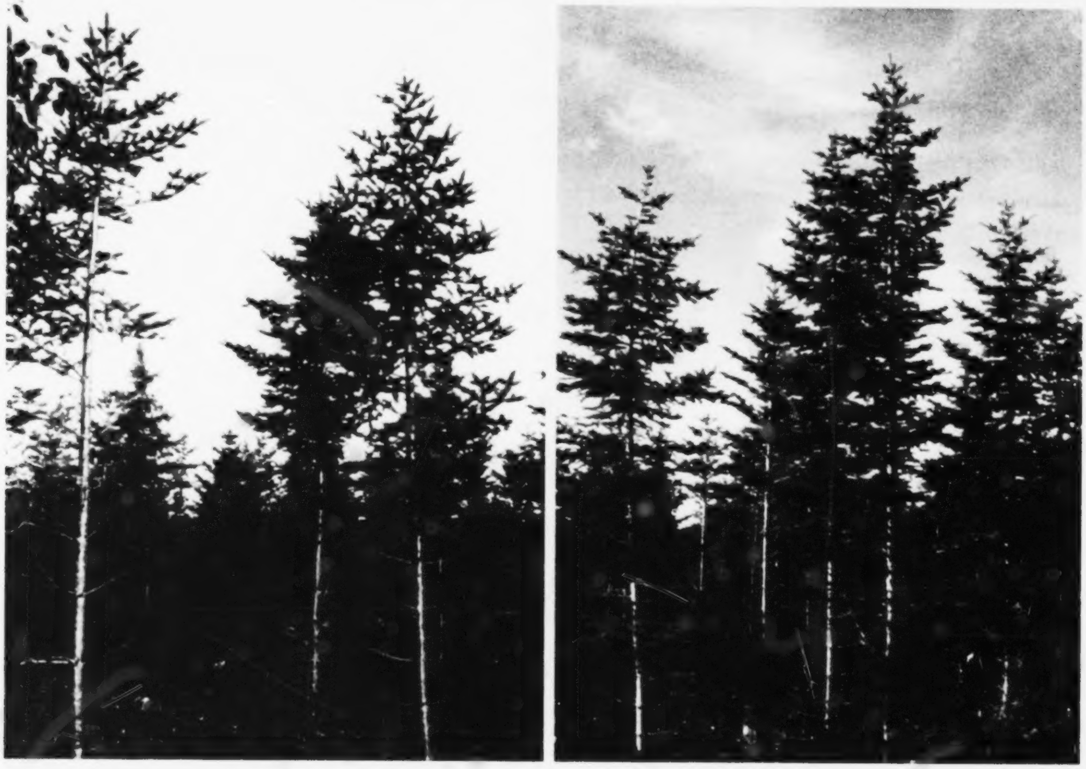

Figure 6. Pre-commercial thinning prescriptions are resulting in abnormal crown to stem and root development subjecting trees to excessive stem sway, root movement and breakage, for many regions in Newfoundland where windy conditions are common.

A tentative listing of the butt rot fungi isolated from the PCT survey is listed in Table 1. The occurrence and type of decay fungi isolated from the PCT plots differed considerably from fungi isolated in natural regenerating stands. One notable difference was the high incidence of *Stereum sanguinolentum* occurring as a butt rot decayer whereas it is normally associated with stem heartrots. *Hypholoma fasciculare* was isolated from root systems of living trees. A common slash/dead wood decayer appearing as a root rot fungus affecting the below ground structural integrity of standing trees. Butt rot fungi from the 2001 survey are in the process of being identified. A comprehensive proposal is being developed to address the concerns regarding the impact pre-commercially thinning is having upon root rot development in treated balsam fir stands in Newfoundland.

Table 1. Butt rot decay organisms isolated from semi-mature pre-commercially thinned balsam fir stands in Newfoundland and their relative occurrence to those isolated from natural regenerated second growth stands (Whitney 1995).

Newfoundland		Ontario	
Fungus	**% trees**	**Fungus**	**% trees**
Stereum sanguinolentum WR	18.8	*Armillaria ostoyae* WR	45.7
Coniophora puteana BR	15.2	*Scytinostroma galactinum* WR*	10.2
Armillaria ostoyae WR	13.8	*Coniophora puteana* BR	9.3
Resinicium bicolor WR*	10.9	*Resinicium bicolor* WR*	7.6
Peniophora A (pseudopini) WR+	8.7	*Stereum sanguinolentum* WR	4.6
Peniophora D WR +	5.8	*Xeromphalina campanella* BR	3.7
Peniophora B (pithya) WR+	4.3	*Perenniporia subacida* WR*	4.3
Hypholoma fasciculare WR+	3.6	*Serpula himantioides* BR	2.4
Perenniporia subacida WR*	3.6	*Inonotus tomentosus* WR	2.2
Tyromyces balsameus BR	3.6	*Tyromyces balsameus* BR	1.5
Scytinostroma galactinum WR+	1.4		

decay type: WR - white rot
BR - brown rot

* indicates black flecks characteristic in decayed wood
+ indicates black flecks present but scattered

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EARLY EVENTS OF INFECTION OF ROOTS OF *PINUS SYLVESTRIS* SEEDLINGS WITH *HETEROBASIDION ANNOSUM* STRAINS OF P-, S-, AND F-INTERSTERILITY GROUPS – SCANNING ELECTRON MICROSCOPY

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SUMMARY

Prepenetration and penetration phenomena on roots of *Pinus sylvestris* seedlings grown *in vitro* after inoculation with P-, S- and F-group isolates of *Heterobasidion annosum* were observed using scanning electron microscopy. There were no differences in the arrangement of hyphae and in the appearance of mycelia formed by the three IS-group isolates. Four types of the root entering by hyphae were observed. In the first group, penetration was achieved through tiny pores formed by enzymatic erosion of cell wall material. In the second, infection structures resembling appressoria were formed by the hyphal tips. In the third, the hyphae penetrated roots *via* holes in eroded areas of cell walls, and in the fourth, by the natural crevices.

Keywords: *Pinus sylvestris, Heterobasidion annosum* P-, S- and F-IS-group strains, prepenetration and penetration phenomena

INTRODUCTION

Although *Heterobasidion annosum* (FR.) Bref. is considered to destroy woody roots, recent findings have shown that this pathogen is also able to infect primary roots of conifers (Asiegbu et al. 1993, 1994; Heneen et al. 1994 a,b; Werner 1990; Werner and Idzikowska 2001). SEM observations on penetration of non-suberized roots of *Picea abies* by hyphae of the S-group isolate were described by Asiegbu et al. (1993). Similar observations on penetration of woody roots of *Picea abies* through broken phellem cells and intact bark was described by Heneen et al. (1994b).

Attachment to the host is presumed to be the first step in host-pathogen recognition and in establishing the parasitic interaction. Surface mucilage and polysaccharides are known to function in adhesion of fungal structures to the root surface (Bracker and Littlefield 1973). It is particularly abundant in the slimy root region, where the cortical cells slough and infection takes place (Werner and Idzikowska 2001). The role of mucilage in adhesion and germination of *H. annosum* spores, superficial colonization and penetration has been reviewed recently by Asiegbu et al. (1998). To date, however, there is no information concerning the differences in the behaviour of mycelia of the three intersterility groups on the root surfaces of host-plants. Since studies on the prepenetration phenomena seem to be of a great importance for a better understanding the host preferences in *H. annosum* complex, a model system has been developed to study the external and internal colonization of conifer roots by the pathogen in uniform *in vitro* conditions.

The objective of this study was to document the differences in the mode of penetration and establishment of mycelia of the P, S, and F isolates of *H. annosum* on the root surfaces of *Pinus sylvestris* seedlings.

MATERIALS AND METHODS

Plant and fungi

Plant material was *Pinus sylvestris* seedlings grown under sterile *in vitro* conditions. *Heterobasidion annosum* was represented by three strains of the P-, S-, and F-intersterility groups (W2, 96043 and 96067).

Growth conditions and inoculation procedure

Sterile pine seedlings were grown under conditions described by Werner and Idzikowska (2001). Two-month-old seedlings were aseptically transferred from test tubes into Petri dishes containing a sterile mixture of perlite and peat (3:1 v/v). Subsequently, they were inoculated with several pieces of mycelial mats of the fungi and incubated in a growth room under fluorescent tubes (Osram L36/W77 Flora) ($100\mu Em^{-2}s^{-1}$) light 18 hours a day, 80% RH at 24:20°C day:night temperatures for 24 hours, 3, 7, and 14 days. Noninoculated seedlings served as the control.

Root preparation for scanning electron microscopy (SEM)

Small pieces (3-5 mm) of apical parts of roots were fixed in 2.5% glutaraldehyde in 0.5 M cacodylate buffer at pH 7.2 for 24 h and postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 h at 4°C. The specimens were then washed in distilled water, dehydrated, and critical point dried in a Balzers CPD-030 unit using CO_2 as a transition fluid. Specimens were then mounted on aluminium stubs and coated with gold (12-15 nm thick) using a Balzers SPD-050 sputter coater. Finally, the roots were observed in a Philips 515 scanning electron microscope at 15 keV.

RESULTS

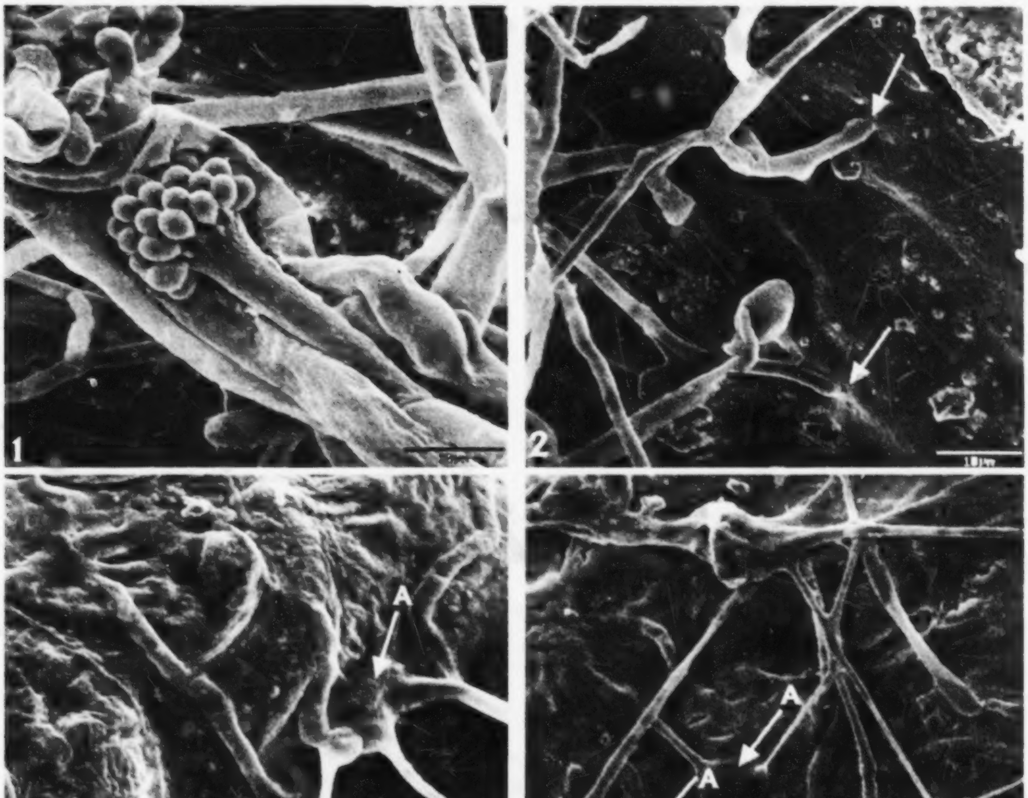
Within 24 hours, elongated and branched hyphae emanating from the inocula were observed on the root surfaces. Penetration sites were detected within 3 to 7 days after inoculation. From days 7 to 14, the number of hyphae increased, forming a compact mat. Numerous conidiophores bearing conidia (Fig. 1) made it impossible to observe the infection points. There were no distinct differences in frequency of the conidiophores formed by the three isolates.

Four types of the root entering by hyphae were observed. In the first group, the hyphal tips perforated the cortical cell wall (Fig. 2). Penetration was achieved through tiny pores, most probably formed by enzymatic degradation of the cortical cell wall. In the second group, a single or several hyphae formed a structure similar to appressorium, which was tightly appressed to the root surface (Fig 3). In most cases, the hyphal tips simply disappeared in the mucilage covering the roots (Fig. 4). The lack of further growth of the hyphae on the root surface may suggest that the penetration was completed. In the third group, an extensive erosion of the cortical cell walls preceded the penetration. The entrance of hyphae into roots was achieved simply through holes in eroded cell wall without formation of infective structures (Fig. 5). In the fourth group, the hyphae entered the roots through crevices between sloughing cortical cells (Fig. 6).

Although there were no differences in the arrangement of hyphae and in the appearance of mycelia formed by strains of the three IS groups, the first mode of penetration was most often observed after inoculation with the P- group strain. The second type was dominating on roots inoculated with the S-group strain, while the third type was the most numerous after inoculation with the F-group stain. The fourth type of root entering by hyphae was observed in similar frequency after inoculation with each strain of the fungus.

DISCUSSION

The results of the study did not provide uncontrovertible evidence of various behaviour of hyphae of the three IS-group isolates of *H. annosum* on pine root surfaces. Nevertheless, the most frequent perforation of the cortical cell walls in a form of a tine pore by the hyphae of P-group isolate, presumably due to enzymatic erosion of cell wall material.



Figs. 1-6. Fig. 1. Conidiophore of *Heterobasidion annosum* with attached conidia (14 after inoculation with F strain). Fig. 2. Direct penetration of hyphae through small pores formed due to enzymatic degradation of the host cell wall. Three days after inoculation with P strain. Fig. 3. Appressorium-like structure (seven days after inoculation with S strain). Fig. 4. Disappearing of hyphal tips in root mucilage (seven days after inoculation with S strain). Fig. 5. Entering the root by hyphae in eroded areas (seven days after inoculation with F strain). Fig. 6. Direct penetration of hypha (D) *via* crevice (arrows) (one day after inoculation with F strain).

Erosion of the wall materials may suggest a high facility to accomplish the penetration of pine roots. In contrast, formation of infective structures may suggest a need for a mechanical force and an enzymatic action to complete the wall perforation. Since the different types of penetration were observed in different frequencies on roots inoculated with each of the IS-group strains and since formation of appressoria-like structures by hyphae of *H. annosum* strain of the S group on *Picea abies* roots was described by Asiegbu et al. (1993), the differences we observed in the mode of penetration completed by the hyphae of the same strain may be related to the differences in structure and/or thickness of the cortical cell walls.

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MOHIEF: MODELLING OF *HETEROBASIDION* IN EUROPEAN FORESTS, A EU-FUNDED RESEARCH PROGRAM

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SUMMARY

This EU-funded research program brings together forest pathologists, forest ecologists, modellers, and forest managers to prepare a prototype decision-support tool enabling potential losses to *Heterobasidion* infection in the range of forest types found in Europe to be estimated. The tool will enable forest managers to predict likely losses to disease and the possible effects of proposed management inputs on subsequent disease development. Different sections of the program will (a) establish the output requirements of the end-users for such a tool, (b) collate the biological and management inputs required, and (c) construct a prototype simulation model, concentrating on adaptability and user-friendliness. End-users will be involved particularly in determining the required model inputs and in testing the preliminary support-tool prototypes. The program is perceived as a data collating and basic model construction prelude to development of a full model and extensive field testing under a future research program.

Keywords: *Heterobasidion*, modelling, decision-support tool, disease management, prototype development

INTRODUCTION

Heterobasidion species are the most economically damaging disease agents affecting coniferous timber production in the north temperate zone; the combined financial impact of the three species of *Heterobasidion* in the 15 member states of the current EU (1998) has been estimated at over Euro 800 million per annum (Woodward et al. 1998). Because of the biology of the causal organism and the fact that it is a disease of forests managed for production, the incidence, and also therefore the economic impact of the disease, increases with successive coniferous rotations (Korhonen and Stenlid 1998; Redfern and Stenlid 1998; Stenlid and Redfern 1998). Effective and ecologically acceptable control methods for this disease would, therefore, be of enormous benefit to the European forestry industries, and the people who rely on forestry for their incomes.

Despite the high economic impact of *Heterobasidion* on European forestry, the justification for controlling this disease on economic grounds is one of the most intractable problems associated with forest management (Pratt 1998). Over the past few decades, information on the infection biology of the disease caused by *Heterobasidion* in coniferous trees has increased enormously (see reviews in Woodward et al. 1998). However, our abilities to predict the incidence of the disease and to control it on sites already infected are limited. In order to justify fully the costs of control, whether currently available chemical, biological or physical methods, or the possible use of host resistance, accurate forecasts of disease development are required. In agriculture, many such disease models are available. Although forest growth forecasting models have been commonplace for many years, and are considered essential in forest management, disease models are uncommon.

The most comprehensive attempt to utilise disease modelling in forest management is the Western Root Disease (WRD) model developed in the 1980's and 1990's for use in North Western America (Frankel 1998) in response to concerns expressed by forester managers over the lack of information on the impact of root diseases on future growth and development of stands. This model was developed through a series of interactive workshops which involved forest root disease pathologists and forest managers, to help predict the impact of root disease caused by *Armillaria* spp. and *Phellinus weirii*, but was soon also adapted for use in stands infected by *Heterobasidion annosum*. In Europe, a number of localised models have been partially and separately developed in the UK (Pratt et al. 1989), Sweden (Vollbrecht 1994) and Finland (Möykkynen et al. 1998). These models were developed to predict the incidence of decayed trees through several rotations with and without intervention control measures. The most advanced model is probably that developed recently in Finland (Möykkynen et al. 1998), which uses sub-models to simulate stand growth, infection of stumps and logging injuries by *Heterobasidion*, vegetative spread of the pathogen in roots and the development of decay. All of the models, however, are based on data from localised sources, and require considerable adaptation to apply in other climatic and forest management conditions.

The overall aim of this work is to develop an efficient, effective user-friendly computer-based model to predict the incidence and future development of *Heterobasidion* infections adaptable to the different coniferous forest ecotypes found in Europe. This paper describes work recently begun in an EU-funded Concerted Action, the aim of which is to gather the data required to construct the basic modelling system, with input from EU countries having different forest types and management systems. The model will then be adapted to the requirements of end-users. The end-product of the work will be software for a working prototype model, focused on the user interface systems. This paper described the methods used in the project, and the likely outcomes.

METHODS

The project is divided into four work-packages, the first three of which will set the parameters for developing and testing the model, and the fourth which develop a model.

1. Output Requirements

This sub-section will examine in detail the output that is required of a model for *Heterobasidion* disease. The principal question addressed in this section is: "*What does the forest manager require in terms of model output*?" The most likely management requirements are:

- estimates for the expected rate of development of *Heterobasidion* in a stand (infections, decay), under given ecological conditions and management scenarios.
- estimates of the extent of losses due to *Heterobasidion*, including growth reduction and mortality of trees, yield reduction expressed in the reduction of saw log timber, pulp wood, and other assortments; volume transfers between timber assortments due to decayed wood; and ecological impact resulting from infection and from control.
- how can control be optimized, for example appropriate methods in different situations, timing of application of controls, economic validity of applying different controls, or maximising financial returns.
- how can thinning and harvesting be optimised, in relation to predicted rates of disease development.
- what are the likely infection rates in new tree rotations, after regenerating an affected stand with alternative tree species.

Managers like to use modelling to answer 'what-if questions' and to optimize forest inputs. What-if questions enable the manager to predict the likely consequences on disease development of a given set of management actions, such as thinning the stand in summer without stump treatments. Managers also want to use the most appropriate treatment schedule for a stand, i.e. the combination of treatments that maximizes the site expectation value or some other objective variable. Answering what-if questions requires that current knowledge of stand development and progress of *Heterobasidion* infections be collected into a computerized simulation program. Optimization requires the program to be linked with a suitable optimization algorithm, which is able to compute the best combination of treatments for a stand.

2. Inputs

The following parameters will be determined for a number of different forest ecotypes, in order to develop inputs to the system: stand development, rate of logging injury, infection frequency and rate of disease development.

- Stand development

Stand development models currently used in different EU countries (e.g. Solberg and Haight 1991; Pukkala et al. 1998) will be reviewed to determine the most appropriate one(s) on which to base the *Heterobasidion* model. Current stand development models, however, are based on different modelling approaches, such as stand level, diameter distribution, distance-independent individual tree or distance-dependent individual tree models.

Tree level stand description and growth modelling may provide the best input data to simulate the dynamics of *Heterobasidion* infection. A stand description in which all trees are known by location provides the best possibility for mimicking the spread of *Heterobasidion* in a stand. Both distance-independent and distance-dependent models can be used with this kind of stand description. However, since it is likely that tree-level models will not be found for all countries, a modelling approach compatible with other growth model types must also be considered. A possible solution to the problem of varying growth models, is to have country-specific sub-models for tree growth. The nature of the sub-models for the infection rate of *Heterobasidion* and its spread in the stand will be common for all European countries, although the values applied in different member states will vary.

- Rate of logging injury

Logging injuries provide a possible route for entry of *Heterobasidion*, particularly *H. parviporum*, into trees. The number of these injuries, including size of wounds, distribution on the trees, and proximity to extraction routes, will be modelled, or existing knowledge converted into suitable models. Current knowledge is very scattered and in varying forms, and may be transformed using 'expert modelling' as proposed elsewhere (Alho et al. 2001; Silvennoinen et al. 2001), or through using the Delphi technique (Dalkey and Helmer 1962).

- Infection frequency

Existing knowledge will be reviewed to find the best possible estimate for the frequency of infection in different conditions. Modelling of expert opinion may be required to find and estimate the dependence of infection rate on various site and environmental parameters.

- Disease development

Parameters such as growth rate of *Heterobasidion* within the tree, the effect of *Heterobasidion* on tree growth, spread of infection within the stand (tree-to-tree, at root contacts), effects of soil type on disease spread and vegetative spread between different tree species will be assessed for inclusion into the model.

3. Preparation of the Prototype Decision-Support Tool and Interface

A prototype model will be developed using existing knowledge where possible, both to combine the model with an optimisation algorithm, and to develop a user-friendly interface. A prototype decision-support tool available in Finland will be used as the initial basis for expanding the system for use in a range of forest ecotypes in other countries of Europe. Information obtained under 1 and 2 described above will be used in the preparation of the prototype decision support tool, as follows:

- Simulation program

A module will be developed which converts the stand data into a form which can be used as model input. The disease simulation program will be linked to forest growth and yield models currently in use in different countries, many of which may need to be reprogramd. The simulation model will be validated both quantitatively and qualitatively.

- Decision support system

The decision support system will be selected and an appropriate optimization tool chosen. These will be combined and user-friendly interfaces developed for testing them with forestry decision-makers.

4. Tests with End-Users

The prototype model will be tested by forest managers in order to obtain feed-back on its usefulness in forestry practice, and on the quality of the predictions and the decision support provided by the system. Testing will begin early during the work on preparation of the prototype, so that the feed-back can be used during the remaining time of the project to improve the prototype model, to expose different options of the decision support system, and to provide experience of the user interface.

End-users to test the prototype system will be the forest managers from the different participating countries. This work-package will be based on the products from work-package 3, and the results of the work-package will be returned to work-package 3 so that appropriate modifications can be made to the prototype model.

DISCUSSION

The development of a European Model for *Heterobasidion* root disease will provide an invaluable tool in the sustainable management of coniferous forests and plantations. Drawing partly on previous models separately developed in the UK, Sweden and Finland, plus the 'Western Root Disease Model' used in North America, new information will be added on the relative frequencies and rates of spread of the three European species of *Heterobasidion* in different host species, and different sites and forest types. The model will enable informed predictions of the chance of infection development in a given location with particular coniferous species and management regimes, and its likely rate of development. Moreover, decisions on appropriate management methods and the application of control procedures will be based on these scientifically justified predictions.

This work will generate the prototype disease modelling simulation, but it is beyond the scope of such a program to exhaustively test the prototype model in the range of forest ecotypes found in Europe, or to carry out the re-programming necessary to fully interface the prototype model onto the range of current forest growth and yield models used in all European countries. A future proposal, therefore, will modify the prototype model, making it adaptable to the forest management systems used throughout Europe.

Feed-back obtained from end-users participating in the project can be used during the project gradually to improve the models, to test different options in the decision support system, and to provide experience of the user interface. End-users will also judge the effectiveness of the system for their own forest management conditions, and the model can be adjusted accordingly.

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INFECTIOUS CYCLE OF *ARMILLARIA OSTOYAE* ON MARITIME PINE STANDS OF DIFFERENT AGES

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SUMMARY

Armillaria root disease is one of the most serious diseases of maritime pine in the South-West of France. Two surveys, conducted at ten-year intervals in the Landes de Gascogne forest, have shown the presence of *Armillaria* mortalities at different stand ages of maritime pine (Levy et al. 1997). The severity of *Armillaria* root disease appears to be different in young stands compared to mature stands. In young stands, disease development and mortality are rapid during the first years after planting. The epidemic then stagnates after the 7-8th year (Lung-Escarmant et al. 1997). Sometimes, in mature stands, disease centres become re-activated after such periods of stagnation. In order to understand this phenomenon, infection paths of *A. ostoyae* at the tree level were compared in 10-year-old and 25-year-old stands. Root infection of 45 trees of each age stand was assessed using a scale of 0 (healthy root) to 5 (dead root with no resin reaction) in order to define an infection class for each tree at time 0. Mortalities were noted every six months for two years and the evolution of root infections was observed at the end of the two year period (time 2).

Mortality of pine trees not or slightly infected at time 0 (classes 0 and 1) were compared and revealed large differences in mean time between initial infection and tree death (infectious cycle) in 10-year-old and 25-year-old pines. The infectious cycle can be quantified as a function of time for mature trees only. Observation of roots after the two year period revealed strong host reactions on young trees (resin reaction, necrosis healing, formation of adventitious roots above the necrosis), but absent on 25-year-old trees.

INTRODUCTION

Armillaria root disease is one of the most widespread diseases of maritime pine in the South-West of France. Two surveys, conducted at ten-year intervals in the Landes de Gascogne forest, have shown the presence of *Armillaria* mortalities at different ages of maritime pine (Levy and Lung-Escarmant 1997). The evolution of *Armillaria* mortality can be very unpredictable and forecasting potential tree loss during the forest stand lifespan remains difficult.

In young stands, disease development and mortality are rapid during the first few years after planting. Later on, the epidemic stagnates after the 7-8th year (Lung-Escarmant et al. 1997). Sometimes, in mature stands, disease centres become re-activated following a stagnation period.

In order to understand this phenomenon, infections paths of *A. ostoyae* on individual trees were compared in 10-year-old and 25-year-old stands. Improved knowledge of the pathogen epidemiology is therefore required before implementing a risk forecast model.

METHODS

Pine stands at two stages of their lifespan were chosen : a juvenile stand (10 years old) and a mature stand (25 years old). Forty-four living trees of each age (4 plots of 11 trees for each stand) were selected in areas of high inoculum pressure (i.e. located less than 6 m from trees recently killed by *A. ostoyae*).

All trees were assessed by clearing the soil from root systems within a 30 cm radius from the trunk in order to examine major roots for infection by A. ostoyae. All roots were assigned a 0 to 5 root condition score based on the presence of *Armillaria* and resin reaction (Table 1A). The presence of other host reactions was also noted (callus and adventitious roots at the margin lesions). The infection level of each tree was calculated (means of root scores) at the beginning of the experiment (T0) and all trees were assigned a 0 to 5 tree condition score (Table 1B).

Table 1. Evaluation of root and tree infection levels.

Rhizomorph	**Resin reaction**	**Fans under the bark**	**Score**		**Infection level***	**Class**
0 *	0	0	0		0	0
1 **	0	0	1		0.1 to 0.99	1
0-1	1	0	2		1.0 to 1.99	2
0-1	1	1 on half of the root	3		2.0 to 2.99	3
0-1	1	1	4		3.0 to 3.99	4
0-1	0	1	5		4.0 to 4.99	5
Table 1A	* 0 = absence	** 1 = presence		Table 1B	* mean of	root scores

Mortalities were noted every six months. The dynamics of the disease was followed in order to evaluate the duration of the Armillaria infectious cycle, i.e. the mean time taken by Armillaria to kill a recently contaminated tree. In our study, trees belonging to class 0 and 1 were considered at the beginning of infection:

- Class 0 (by default) = no *Armillaria* observed on large roots;
- Class 1 (by excess) = presence of either rhizomorphs or resin reaction on a few roots.

The duration of the infectious cycle (in months) = ${}_0^N\Sigma\ (n_i\ xT_i)/\ N$ with n = number of dead trees; T= survey period (in months) ; N = total number of trees in class 0-1.

At the end of the two year period (T2), the infection level of trees still alive was re-assessed in order to investigate factors contributing to survival.

RESULTS

Evaluation of Armillaria infection on individual trees

76% of the maritime pine trees analysed had belowground infections without above-ground symptoms whatever the infection level and the stand age. The trees had very variable infection levels, distributed into all the different infection classes regardless of age (Figure 1).

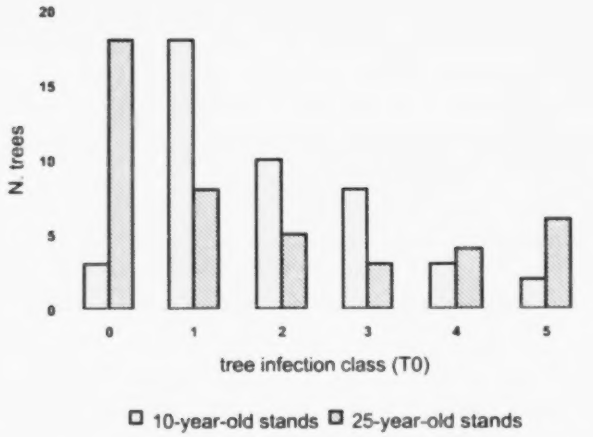

Figure 1. Distribution of trees in each infection class at the beginning of the experiment (T0).

The infection level, which varies greatly between trees, is related to the amount of secondary inoculum present near the tree. In 10-year-old trees, we found a significant positive correlation between the number of surrounding dead trees and the infection level (R= 0.23, p = 0.0001). In 25-year-old trees, there was a significant negative correlation between the distance from the nearest dead tree and the infection level (R = -0.375, p = 0.001). Twenty-one trees in young stands and 26 trees in mature stands were at the beginning of infection (class 0-1).

Dynamics of Armillaria root disease and evaluation of the infectious cycle duration

In mature stands, the majority of trees (78.5 %) of different infection levels died within two years. In young stands, few trees (14.6%) of initial infection level > 2 died within two years (Figure 2).

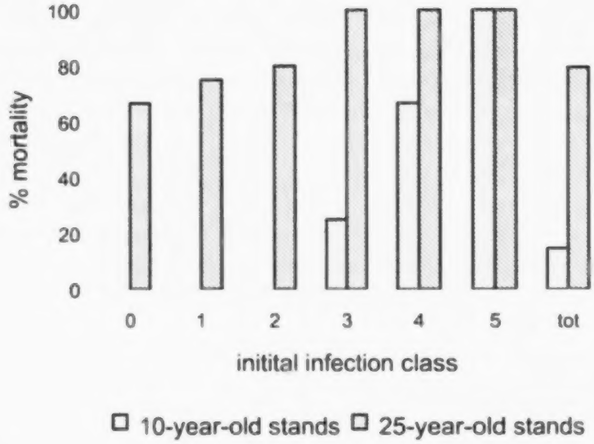

Figure 2. Evolution of the disease in two years: percentage of trees dead after two years in the different initial infection classes.

An initial evaluation of the infectious cycle duration can be made for the mature stand only, as the majority of trees (73%) recently contaminated (class 0-1) died within two years. In the mature stand, the current average duration of the infectious cycle is 18.3 months.

Evaluation of infection level of trees still alive after two years

In young stands, we observed a regression in the infection level of trees with low initial infection (classes 0-2) (Figure 3). It can be explained by the presence of callused lesions and adventitious roots at the margin of the lesions on the majority of roots. In mature stands, tree infection levels increased whatever the initial tree infection level (Figure 3). In this case, 81% of roots were infected two years after the evaluation of the initial infection level and the majority of lesions were active (presence of mycelium fans without resin reaction).

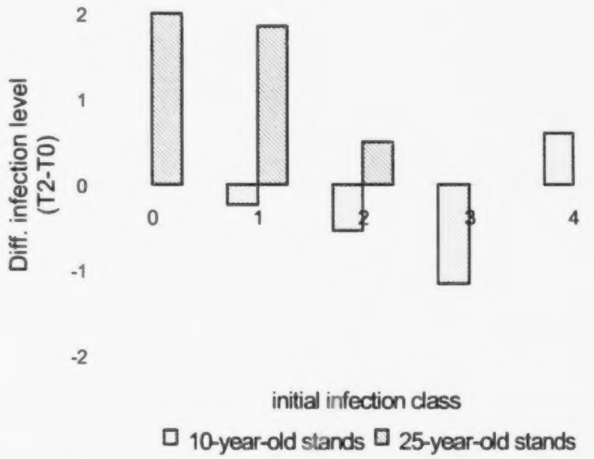

Figure 3. Evolution of tree infection level after two years (T2- T0) in trees still alive.

CONCLUSION

This study shows how variable the dynamics of *Armillaria* can be from one pine stand to another. It appears to depend on host defence systems. Such responses would explain the epidemic stagnation in 10-year-old stands. However, surprisingly, defence systems appear much less effective in older trees. This study requires further investigation in more sites with varied epidemic dynamics.

More knowledge is also required concerning the role of host tree (age, genetics), pathogen (aggressiveness, rhizomorph production capacity) and environment on the host reactions in order to better define the evolution of the *Armillaria* infectious cycle at different stages of the pine lifespan in the Landes forest.

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EARLY DEVELOPMENT OF *HETEROBASIDION* ROOT ROT IN YOUNG NORWAY SPRUCE STANDS

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SUMMARY

Heterobasidion infection of planted and advance-growth regenerations of Norway spruce was studied on old spruce sites in southern Finland. The study plots were established on sites where the previous spruce rotation had been infected by *Heterobasidion* (mainly *H. parviporum*). The occurrence of decay in regeneration trees, as well as in stumps and trees of the previous generation, was investigated. *Heterobasidion* was isolated from cases of decay, and the genotypes of the fungus were identified. The average number of regeneration trees infected by *Heterobasidion* per decayed stump in the previous rotation was 4.5 in the advance regeneration and 1.7 in the planted stands. At least 53% of the *Heterobasidion* infections in advance-growth spruces and 71% in planted spruces originated through direct growth of the fungus from the previous spruce generation. The origin of the genets, which were not connected to the old rotation, could not be identified with any certainty. However, the close genetic relationship between many of the isolates suggests spore infection from one or a few local genets. The proportion of broadleaf wildings in the regeneration did not affect the disease frequency of the young spruces. According to these preliminary results, the frequency of *Heterobasidion* infection tends to be higher in advance spruce regeneration compared to planted stands. On the other hand, the disease progresses more rapidly in the wood of young planted spruces.

Keywords: *Heterobasidion* root rot, *Picea abies*, regeneration, planting, advance growth, genet

INTRODUCTION

Heterobasidion root rot is an important factor to be considered when regenerating Norway spruce (*Picea abies*) stands in southern Finland. On old spruce sites, where *H. parviporum* Niemelä & Korhonen (the S type) is the main cause of butt rot, changing the tree species is the best method to control decay losses in the subsequent tree generation. However, the use of both Scots pine (*Pinus sylvestris*) and silver birch (*Betula pendula*) is often questionable because of unsuitable soil properties or a high risk of elk browsing damage. In such cases, Norway spruce may be the preferred tree species even on infested sites, and proper silvicultural measures are needed to keep the losses caused by *Heterobasidion* at a reasonably low level.

Relatively little is known about the effect of regeneration method on the spreading of *Heterobasidion*, and the information that is available is partly contradictory. Some studies suggest that naturally regenerated spruces are less often and less severely affected by root rot than planted spruces (e.g. Weissen 1981, Graber 1996). In contrast, there is some evidence that advance growth of Norway spruce, which has developed under a spruce overstorey, can be seriously infected by *Heterobasidion* (Kangas 1952, Semenkova 1971).

In an earlier study carried out in southern Finland, an average 21% (max. 68%) of the untended regeneration spruces growing under a diseased spruce overstorey had *Heterobasidion* infection in their root system (Piri and Korhonen 2001). The advance regeneration may thus promote the spreading of *Heterobasidion* root and butt rot from the previous to the next spruce generation. As regards planted regenerations, recent Nordic studies dealing with the incidence of *Heterobasidion* root rot in unthinned spruce stands did not show any correlation between the disease incidence in consecutive spruce rotations. Even on heavily infested sites, the average proportion of planted spruces with visible decay at stump height was less than 10% at the first thinning (Vollbrecht and Stenlid 1999, Rönnberg and Jørgensen 2000).

A series of investigations was started in Finland in 1998 in order to determine the early development of *Heterobasidion* root rot in young unthinned spruce regenerations established on infested sites. In this report we present preliminary results concerning the early infection of planted spruce, and compare the results with those obtained from an earlier study on advance spruce regenerations (Piri and Korhonen 2001).

MATERIAL AND METHODS

Sample plots

The study material consists of 17 sample plots established in advance spruce regenerations (Piri and Korhonen 2001), and 21 plots established in planted spruce stands. The plots were located on 15 old forest sites in southern Finland. The previous rotation had been a pure or mixed Norway spruce stand infected by *Heterobasidion*. On the advance-growth plots the spruce understorey had developed under the infested overstorey. Three control plots in advance regenerations and three plots in plantations were located in a healthy part of the stand with no visible signs of infection in the earlier tree generation. The age of the advance-growth spruces varied from 14 to 44 years (mean 26 years), average height from 0.4 to 12.5 m (mean 2.8 m), and density from 2200 to 53 400 trees/ha (mean 11 800). On the planted plots, the sites were clear cut and planted after site preparation with 3- to 5-year-old Norway spruce seedlings at 2 x 2 m spacing (2500 plants per ha). The age of the planted stands varied from 2 to 23 years (mean 15), and the average height from 0.4 to 9.5 m (mean 4.8). The proportion of admixed tree species (mainly *Betula pubescens*, *B. pendula*, *Sorbus aucuparia* and pine wildings) on the advance-growth plots ranged from 4.6 to 69.4% and on the planted plots from 4.2 to 88.2%. According to the Finnish classification, the sites were of the *Myrtillus* or *Oxalis-Myrtillus* forest site types. No thinnings had been carried out in the studied regenerations.

Measurements and sampling

All the standing trees (height > 0.3 m) and stumps on the plots were mapped and measured. Tree species, origin (planted or natural), height, diameter at breast height (1.3 m) or the stump diameter were recorded. The presence of *Heterobasidion* in the present and previous tree generation was determined by sampling all the coniferous trees and stumps on the plot. In addition, the old stumps of the previous generation were mapped and sampled on a ca. 5 m-wide buffer zone outside the plot boundary.

The root system of all the advance-growth spruces and planted spruces younger than 20 years of age were dug out, washed, and examined for infections (Piri and Korhonen 2001). In plantations over 20 years old, a wood sample was taken aseptically with an increment borer from each main root and from different sides of the butt of the tree. Upward extension of the decay in the infected stems was measured. The old stumps (butt and major roots) were sampled by means of an axe and saw. The proportion of stump wood colonized by *Heterobasidion* was estimated visually on the surface of the stump and in the main roots. The wood samples were cultured on agar medium, *Heterobasidion* isolated, and the fungal genotypes were identified with the aid of the somatic compatibility reaction.

Pearssons's product-moment correlation analysis and stepwise multiple regression analysis were used to study the degree of association between the infection frequency in regeneration as the dependent variable and the tree and stand characteristics as independent variables. All the test were considered significant at a probability of α = 0.05.

RESULTS AND DISCUSSION

A total of 2199 advance-growth spruces and 402 planted spruces in the regenerations were investigated; 21.1% of the former and 17.7% of the latter were infected by *Heterobasidion*. *H. parviporum* was by far the predominant species; the proportion of *H. annosum* (Fr.) Bref. s. str. of all *Heterobasidion* isolates was only 1.7%.

The decay frequency was generally higher in advance regenerations than in planted stands of the same tree size (Fig. 1). In both types of regeneration, the decay frequency correlated positively with tree size, age of the regeneration, and frequency of old infected stumps and trees on the plot. In advance regeneration, a weak negative correlation was found between the disease frequency and the regeneration density. The other stand characteristics did not show significant correlation with the root rot frequency in the regeneration. When combined, the two variables "tree size" and "decay incidence of the previous rotation" explained 67% of the variation in disease frequency in the advance regenerations and, according to the preliminary results, about 57% of the variation in the planted stands.

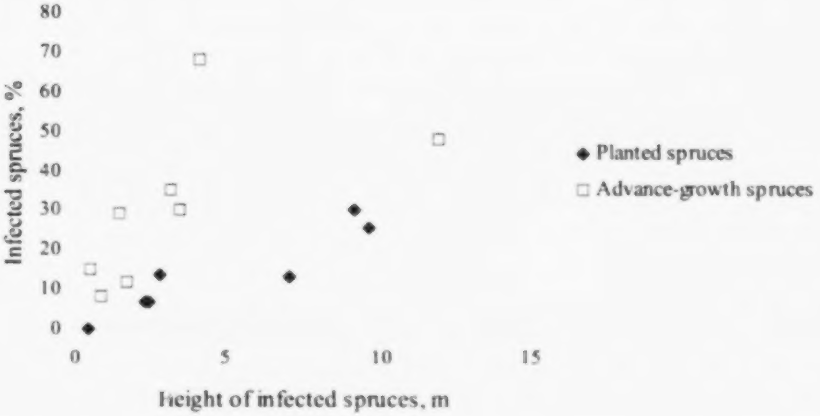

Figure 1. Frequency of the infected regeneration spruces in relation to tree height.

The average number of regeneration trees infected by *Heterobasidion* per decayed stump of the previous rotation was 4.5 in the advance regenerations and 1.5 in the planted stands. 53.3% of the infected advance-regeneration spruces and 71.0% of planted spruces were infected by a genotype that was also isolated from the overstorey, indicating that the fungus has spread vegetatively through root contacts from the old generation. The maximum number of trees and stumps infected by one genotype in the advance regeneration was 46 trees (13 from the previous and 33 from the present tree generation), and in the planted regeneration 15 trees (five old stumps and 10 planted spruces).

The origin of infections not connected to the old stumps, could not be identified. However, a close genetic relationship (unclear demarcation lines between paired mycelia) was frequently observed between genets including a single advance-growth spruce. This relatedness is probably a result of multiple infections by spores originating from one genet. The same phenomenon was not observed in the planted spruce stands. A few advance-growth spruces were also infected on two control plots where the possibility of disease transfer through root contacts was slight.

In contrast to our results, Rönnberg and Jørgensen (2000) did not find any correlation between the disease incidence of two successive tree generations in planted spruce stands in Denmark. Compared to Finland, decomposition of old stumps is faster in Danish conditions, which may restrict the vegetative spread of

Heterobasidion to the next rotation. Furthermore, the common occurrence of other decay fungi, *Armillaria* spp. and *Resinicium bicolor*, in old spruce stumps in Denmark may prevent the spread of *Heterobasidion*. Plots infected by *Heterobasidion* were selected for our study, and other decay fungi occurred only sporadically in old stumps. On the other hand, the spore infection of healthy stumps at clear felling and subsequent transfer of *Heterobasidion* to the planted spruces has also been suggested as a factor that can diminish the correlation in decay incidence in successive spruce generations (Rönnberg and Jørgensen 2000). In our study, all the cuttings had been carried out in winter and infection of healthy stumps by spores is unlikely.

Although the advance-growth spruces were several years, even decades, older than the planted spruces of the same size, most of the *Heterobasidion* infections (78.7%) were restricted to a small part of the root system. The infections in the planted stands were more advanced: in 80% of the trees the decay had reached the stem, and in 22- and 23-year-old planted trees the mean upwards extension of decay in the stem was 140 cm and 230 cm, respectively. However, the difference in decay volume between natural and planted trees may diminish or disappear after release cutting, when the advance growth reaches the growth rate of planted trees.

According to our preliminary results, the frequency of infected trees is somewhat higher in advance regenerations than in planted stands on sites heavily infected by *Heterobasidion*. One explanation for this could be that advance-growth spruces are more susceptible to primary spore infection than planted spruces. Because the young planted spruces are predominantly infected from old stumps via root contacts, a mixed plantation favouring Scots pine or birch groups in the infection centres could be one recommendable alternative when regenerating spruce stands infected by *H. parviporum*.

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PRELIMINARY STUDY ON THE SURVIVAL AND SPREAD OF *ARMILLARIA MELLEA* IN MULCHES IN GARDENS

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SUMARY

The use of organic mulches in UK gardens has increased in recent years for water conservation purposes and weed suppression. The most common organic mulches used in gardens are bark- and wood-chips. These are used around shrubs, trees or on herbaceous borders. If the material used is fully composted, weed seeds, pests and diseases present will be killed but in many cases the chips are not fully composted when used. One of the most important root diseases in gardens in the UK is honey fungus (*Armillaria mellea*) and one of gardeners' concerns is the possible role of organic mulches in the spread of the fungus. The aim of this project is to investigate the ability of *A. mellea* to survive and grow in mulches. *Pinus silvestris* wood-chip and bark-chip mulch were tested. Containers filled with soil and topped up with a 7 cm layer of mulch were inoculated with three different sized inocula of *A. mellea*. After 12 months, the containers were harvested and rhizomorph length and dry weight were measured. Rhizomorphs were present in all the inoculated containers. The maximum length of rhizomorphs, 85 cm, was found in the wood-chip mulch. However, when the data was analysed using Genstat 5 (fourth edition) it was concluded that after 12 months there was no difference in colonization between wood- and bark-chip mulch and there was no difference in the rate of the colonization process between inoculum sizes.

Keywords: *Armillaria mellea*, mulch, *Pinus silvestris*, colonization

INTRODUCTION

Mulches are used in gardens around shrubs, trees or on herbaceous borders. The use of mulches in gardening in the UK has increased in recent years for water conservation, weed suppression and ornamental purposes. Mulches can help to conserve soil moisture, increase water permeability, control weeds and other competing vegetation, replenish organic matter and nutrients to the soil, insulate the soil and also they can be attractive. Some of the common mulches used in gardens are bark- and wood-chips, gravel, cocoa shells, manure and compost. Gardeners can purchase these materials from local garden centres or specialist shops but in many cases they used their own garden shredded material as mulch. This helps recycling garden material. This homemade mulch may include ground or chipped *Armillaria mellea* infected tree stumps. In some cases these chippings may be used as mulch partially composted or even without being composted. They are more usually composted for about four weeks before they are used, but in these cases it is not clear if the temperature rise during the process of composting is enough to kill weed seeds and to eliminate any pests or diseases present. It will take between 3 and 12 months for the mulch to be fully composted depending on the material (Webber and Gee 1996). Also, tree stumps may sometimes be chipped and used as mulch without being aware of honey fungus infection. One of gardeners' concerns is the survival and spread of *A. mellea* in mulches and also the effect of mulching on the development of *Armillaria* root disease. The aim of this research was to answer these questions.

MATERIAL AND METHODS

The first trial was set up to study the survival and spread of *A. mellea* in mulches. The experiment consisted of 72 containers (5 l) filled with soil and topped up with a 7 cm layer of mulch. The mulch consisted of 3.5 and 5 cm long *Pinus silvestris* wood-chips and bark-chips.

The mulch (bark- and wood-chips) in the containers was inoculated with *A. mellea* using infected hazel (*Corylus avellana*) wood segments by the method described by Guillaumin and Rykowski (1980). Two different inoculum sizes were used, 1.5 cm long by 1.5 cm diameter and 5cm long by 1.5 cm diameter.

The trial ran for 12 months and was carried out in a glasshouse. The temperature of the air and the inside of the containers was monitored during this period. The water content was adjusted to 50% of saturation capacity and the containers were watered by weight two or three times a week depending on the season.

RESULTS

After 12 months, the containers were examined and rhizomorph length and dry weight were measured. Rhizomorphs were present in all the containers inoculated with *A. mellea*. The rhizomorphs were about 0.1 cm in diameter, reddish when young and turning black with age. They all had a similar dichotomous branching pattern.

Mycelium was observed in both wood- and bark-chips. In the case of bark-chips, the mycelium was observed between the plates of the bark. In the case of the wood-chips, the mycelium was observed on the surface of the wood. The amount of mycelium was not measured.

Rhizomorphs were extracted from the mulch by removing carefully the bark- and wood-chips and measured. No rhizomorphs were present in the pots containing no inoculum. This was consistent in both bark-chip mulch and wood-chip mulch. The maximum length of rhizomorphs, 85 cm, was found on wood-chip mulch.

The experimental design consisted of randomised blocks with 10 replications and analysis of variance using Genstat was performed for the rhizomorph length and dry weight data. The difference between means was not significant.

DISCUSSION

There was high variability in the results obtained from the trial. The amount of rhizomorphs measured from a particular treatment in the different units varied considerably. In the treatment of wood-chips inoculated with 1.5 cm inoculum, the range of rhizomorph was 1 - 85 cm.

It can be concluded from the trial that *A. mellea* can spread and survive in bark- and wood-chip mulch. It can be concluded from the statistical analysis that there was no difference in the rate of infection between inoculum sizes and there was no difference in colonization between wood- and bark-chip mulch. These conclusions were not expected at the beginning of the trial where it was expected that bigger inoculum will produce more rhizomorphs. Also, the wood-chip mulch was expected to be a better medium for *A. mellea* growth due to the better retention of moisture by the wood-chips.

FUTURE STUDIES

The second trial is now running to determine if *A. mellea* can survive in mulch after the inoculum has been removed and observe any wood or bark degradation. The third trial also now running is to see the effect of mulching on the *A. mellea* infection process. The final trial will study the risk of infection of healthy plants using already infected mulch.

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SCHADE LAKE ROOT DISEASE SURVEY

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INTRODUCTION

Severe root disease was identified on upland black spruce sites during pre-harvest surveys and an aerial reconnaissance flight in the Schade Lake operating area. This area is within the Porcupine Provincial Forest in western Manitoba. A cut and leave harvest system was planned for this area.

An intensive root disease ground survey was carried out in the autumn of 2001. The objective of the survey was to determine the current extent of the root disease, the current volume loss, and to project losses over the next several years.

METHOD

GPS (Trimble GeoExplorer 3 and Garmin 76) technology was employed to carry out the root disease survey. Root disease centers (> 10 metres in diameter) were mapped along parallel lines spaced 75 meters apart. Disease centres were assumed to be circular. ArcView GIS 3.2 software was used to calculate the infested area from the circular polygons.

An inventory cruise was carried out, placing prism plots in disease centres and in uninfested areas throughout the operating area. This data was used to calculate volume loss due to the root disease.

Volume Loss Calculations

In order to calculate a volume loss due to root disease, it was necessary to estimate the potential volume (volume in the absence of root disease), the volume within the infested areas, the volume in the uninfested areas and the current volume on the site.

The potential volume was estimated by multiplying the volume per ha in the uninfested area (mean volume per ha from the uninfested prism plots) by the total number of ha for the entire survey area. The infested volume was estimated by multiplying the volume per ha in the infested areas (mean volume per ha from the infested prism plots) by the number of ha infested. This volume was then reduced by 8% to account for butt rot in the living trees within the infested areas (Whitney 1976). This value is the mid-point (for mature black spruce) between actual butt rot, (4%) and the Ontario scaled cull (12%). The uninfested volume was estimated by multiplying the volume per ha in the uninfested areas by the number of ha uninfested. The current volume was estimated by adding the infested volume and the uninfested volume. The volume loss was estimated by subtracting the current volume from the potential volume.

In order to predict the volume losses into the future, the infested area was expanded, over a fifteen-year period using an annual spread rate of two metres per year (Whitney and Dumas 1994) and an annual mortality rate of 3.5% (Whitney 1976). The volume loss for each subsequent five-year period was then calculated using the method described above.

RESULTS

Root disease centres were found extensively throughout the surveyed area. Armillaria root rot, white pocket stem rot, brown cubical butt rot, tomentosus root and butt rot and yellow stringy butt rot were present within the disease centres. These organisms resulted in breakage of lateral roots or the main stem.

The majority of the volume loss was due to tree mortality and growth loss. The difference in the gross merchantable softwood volume between the infested and uninfested areas was 102 m^3 per ha (infested volume: 174.4 m^3 per ha, uninfested volume: 276.7 m^3 per ha). Cull due to butt rot accounted for only 1.7% of the volume loss. The area infested was 253 ha (34%) over the entire survey area. The volume loss was 29,442 m^3, which was 14% of the potential volume (205,180 m^3). Projections over the fifteen-year period estimated the infested area to almost double in size to 467 ha (63% of the total area) and the volume loss to increase to 63,263 m^3 or 31% of the potential volume of the surveyed area (Table 1).

DISCUSSION

The cut and leave harvest system plans for the second cut of the operating area to be in 10 to 15 years. By that time, the volume loss was projected to increase to 50,593 m^3 in 2011 and 63,363 m^3 in 2016, an increase of 72% and 115%, respectively, of the current loss (29,442 m^3). Therefore, the cut and leave plan was abandoned and the harvest accelerated in order to reduce the projected volume loss.

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Table 1. Root disease volume loss projections for Schade Lake operating area (summary for all blocks).

Area: 741 ha **Potential Volume: 205,180 m^3**

Year	Mean age	Infested area (ha)	% area infested	Block volume (m^3)	Volume loss	% volume loss*
2001	90	253	34%	175,738	29,442	14%
2006	95	324	44%	166,097	39,083	19%
2011	100	399	54%	154,587	50,593	25%
2016	105	467	63%	141,917	63,263	31%

* % Volume loss = volume loss / potential volume

GROWTH REDUCTION OF DOUGLAS-FIR DUE TO NON-LETHAL INFECTION BY *ARMILLARIA OSTOYAE*

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SUMMARY

In an 18-year-old Douglas-fir plantation, 150 trees were removed from the soil within 10 meter radius plots using a pop-up spacer and the root systems were surveyed for lesions. Height was measured annually as far back as possible. Basal area, height, and volume were calculated at age 10 and at final volume by digitizing cross-sectional disk areas at 0, 0.3, 1.3 and then every 2 m along the stem. Each root lesion was dated by using the presence of traumatic resin canals near the lesion from which infection intensity was then determined (percentage of infected roots arising from the root collar).

Losses in final height were modeled using initial height at age 10 before infection (to account for size differences before infection), years since the oldest infection ($p=0.72$) and infection intensity ($p=0.07$) amounting to an 11% reduction in the worst case. Basal area losses were modeled using the same model as final height except for basal area at age 10, and losses occurred as an interaction between basal area at age 10 and years since infection ($p=0.03$ for an average of 26% loss). Volume losses followed a similar pattern with an interaction between volume age 10 and the years since infection ($p=0.0007$ $R^2=0.72$), and ranged from 20% for small trees to 40% for larger trees after 8 years of infection; infection intensity was not significant ($p=0.38$).

This result suggests that even lightly infected trees can lose volume once they are infected, that faster growing trees lose proportionately more volume, and that radial growth is most affected.

LEPTOGRAPHIUM SPECIES AND THEIR VECTORS AS COMPONENTS OF LOBLOLLY PINE DECLINE

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Leptographium species are associated with root-feeding bark beetles and weevils that attack living pine trees and are commonly present during pine decline and mortality throughout the United States. The biological basis for these relationships is variable, and the relative importance of the beetles and fungi in the death of the trees is still unclear. In the southeastern US, loblolly pine stands affected by root disease are more likely to contain *Leptographium* species within their root systems and appear to be more vulnerable to attack by southern pine beetle than apparently healthy stands. A study to determine the role of *Leptographium* species and their vectors in the etiology of loblolly decline has been initiated. A series of plots in Alabama have been selected which exhibit a range of decline symptoms, and *Leptographium* species have been isolated from the roots and soil of the loblolly trees as well as trapped insects. Soil samples have also been taken from each plot for analysis of physical and chemical characteristics. Above-ground symptoms have been assessed in accordance with Forest Health Monitoring standards. We have found that *Leptographium* can be isolated more frequently from public lands than from privately owned lands, and that there is an inverse relationship between isolation of *Leptographium* and crown density. We are currently identifying the species of *Leptographium* involved and initiating various inoculation studies to elucidate the biological relationships of *Leptographium*, insect vectors, and loblolly decline.

CHARACTERISATION OF PHENYLALANINE AMMONIA LYASE PRODUCTION FOLLOWING CHALLENGE OF SITKA SPRUCE WITH *HETEROBASIDION ANNOSUM*

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In order to characterise the effect of challenge with *Heterobasidion annosum* on phenylpropanoid metabolism in Sitka spruce, phenylalanine ammonia lyase (PAL; EC 4.3.1.5) was purified in bulk from young shoots and needles of mature field-grown trees using an aqueous two-phase system (ATPS) and ion-exchange chromatography. It was necessary to include the ATPS to reduce the interference effect of carbohydrates during protein purification. Western blotting and immunoprecipitation were applied to examine the purified PAL. Several peaks of PAL activity were detected in DEAE-cellulose fractions using a NaCl gradient for elution, suggesting the presence of a number of PAL isoforms in spruce. A DNA probe, based on a Sitka spruce *pal* gene sequence was cloned by PCR and used to probe spruce genomic DNA in Southern blots. Induced *pal* mRNA transcripts were examined in root tissues and embryogenic suspension cultures using a quantitative Northern blotting method.

The results suggest that differential regulation of PAL genes may occur in Sitka spruce, with individual genes having different functions in development and the response to fungal attack.

DISTRIBUTION OF THE *HETEROBASIDION ANNOSUM* INTERSTERILITY GROUPS IN POLAND

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Study material comprises 205 *H. annosum* isolates from 30 different localities. Pathogen was isolated from *Pinus sylvestris, Picea abies, Betula pendula, Abies alba, Larix decidua, Larix polonica,* and *Fagus sylvatica.* Most of the *H. annosum* isolates belonged to the P group. This group was most common on pine and birch. The S group infected Norway spruce and European fir. The F group was found in the south of Poland in the mountains, but it was also noticed in lowland localities at the northern range of fir in Europe. The occurrence of IS groups covers its hosts' natural range. The pine type causes damage in pine stands mainly in the first and second rotations on former arable lands. The S group causes damage in Norway spruce stands, mostly in the 50-80 stands. It could also be found in fir stumps. The fir type is a very weak pathogen or saprothroph. No serious damage was caused by this group in the fir stands. It could also colonise spruce stumps or laying logs.

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